Biochemical and electrophysiological markers predictive of return of spontaneous circulation and post-resuscitation outcome

Thesis

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Biochemical and electrophysiological markers predictive of return of spontaneous circulation and post-resuscitation outcome

Thesis submitted by the student

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Conflict of Interest

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ABSTRACT

The majority of patients resuscitated from cardiac arrest (CA) subsequently die due to post-cardiac arrest syndrome (PCAS), whose mechanisms are only partially understood. We adopted an approach of untargeted/targeted plasma metabolomics in rats to identify metabolites involved in the mechanisms of PCAS to be tested as predictors of outcome. Activation of the kynurenine pathway (KP) for tryptophan (TRP) degradation was demonstrated in rats, pigs and in a small cohort of patients. Decreases in TRP occurred during the post-CA period and were accompanied by significant increases in KP metabolites, 3-hydroxyanthranilic acid (3-HAA) and kynurenic acid in each species, that persisted up to 3-5 days post-CA ($p<0.01$). KP metabolites changes were significantly related to the severity of myocardial and cerebral injuries and survival. Finally, when tested in 155 patients resuscitated from CA, KP metabolites were significantly higher in patients with poor outcomes. The quality of chest compression (CC) is another major issue for cardiopulmonary resuscitation (CPR) success and survival. The decision whether to interrupt CC to deliver a defibrillation (DF) is difficult. The potential benefit of a DF guided by a real time ventricular fibrillation (VF) waveform analysis would maximize DF success, minimize CC interruptions and myocardial damage by repetitive and unnecessary DFs. We evaluated amplitude spectrum area (AMSA) as predictor of DF outcome in two large databases of out-of-hospital VFs, from US (609 patients) and Italy (1.617 patients). AMSA was significantly higher prior to a successful DF than prior to an unsuccessful one ($p<0.0001$). Thresholds for prediction of successful and unsuccessful DFs were 16-17 mV-Hz for success and <7 mV-Hz for failure, with a positive predictive value of 80% and a negative predictive value of 97%. AMSA was a better predictor of DF outcome (AUC 0.86, $p<0.0001$) compared to other VF parameters, i.e. amplitude and frequencies. In conclusion, AMSA would be a useful tool for guiding CPR.

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MAJOR ABBREVIATIONS

3-HAA, 3-hydroxyanthranilic acid

AED, automated external defibrillator

AMI, acute myocardial infarction

AMSA, amplitude spectrum area

ATP, adenosine triphosphate

AUC, area under the curve

BBB, blood–brain barrier

BLS, bystander initiated life support

BNP, brain natriuretic peptide

CA, cardiac arrest

Ca^{2+}, calcium ion

CC, chest compression

CF, centroid frequency

CI, confidence interval

CK, creatine kinase

CO, cardiac output

COP, cardioversion outcome prediction

CPC, Pittsburgh cerebral performance categories

CRP, C reactive protein

CPP, coronary perfusion pressure

CPR, cardiopulmonary resuscitation

cTn, cardiac troponin,

CTR, control

DF, defibrillation

DFA, detrended fluctuation analysis

DmF, dominant frequency

ECG, electrocardiogram

EDTA, ethylenediaminetetraacetic acid

EF, ejection fraction

$EtCO_2$, end-tidal CO_2

FFT, Fast Fourier transform

FPR, false positive rate

HPLC, high-performance liquid chromatography

HR, heart rate

hr, hour

hs-cTnT, high sensitivity cardiac troponin T

ICU, intensive care unit

IDO, indoleamine 2,3-dioxygenase

IL, interleukin

in, inch

I_{NaL}, late Na^+ current

IQL, instrumental Quantification Limit

IQR, interquartile range

K^+, potassium ion

KP, kynurenine pathway

KYN, l-kynurenine

KYNA, kynurenic acid

LAC, logarithm of the absolute correlations

LAD, left anterior descending coronary artery

LC, liquid chromatography

LOQ, limits of quantification

LV, left ventricle

MAP, mean arterial pressure

MDF, median frequency

MdS, median slope

min, minute

MMP, matrix metalloproteinase

MNF, mean frequency

MnS, mean slope

MRM, multiple reaction monitoring

MS, mass spectrometry

Na^+, sodium ion

NDS, neurological deficit score

NMDA, N-Methyl-D-Aspartate

NO, nitric oxide

NPV, negative predictive value

NSE, neuron specific enolase

OR, odds ratios

ORA, over representation analysis

PA, picolinic acid

PCA, principal component analysis

PCT, procalcitonin

PF, peak frequency

PPA, Peak-to-peak amplitude

PPV, positive predictive value

PSA, power spectrum area

PSD, power spectral density

QA, quinolinic acid

RMS, root mean square

ROC, receiver operator characteristic

ROS, reactive oxygen species

ROSC, return of spontaneous circulation

S-100b, protein S-100 beta

SAP, systolic blood pressure

SAPS, simplified acute physiology score

ScE, scaling exponent

sec, second

SOFA, sequential organ failure assessment score

TH, therapeutic hypothermia

TNFa, tumor necrosis factor alpha

TRP, tryptophan

VF, ventricular fibrillation

VT, ventricular tachycardia

SUMMARY

Presentation to the study design and thesis reading

The present thesis includes both experimental and clinical studies directed to discover and/or validate biochemical and electrophysiological markers predictive of return of spontaneous circulation and post-resuscitation outcome.

In order to achieve such aims, the student has performed two separate studies concurrently. The first series of studies was directed to discover and investigate experimentally and clinically new *circulating biomarkers* predictive of post-resuscitation outcome, while the second series of study validated *ECG-derived biomarkers* predictive of successful resuscitation in two large databases.

Indeed, these two studies focused on different "markers" targeting the two main aspects of cardiopulmonary resuscitation, namely: successful resuscitation and long-term outcome. Both the studies therefore concurred to the same goal of identify new tools to predict cardiac arrest outcome and are therefore presented together in this thesis as a single study with an introduction, materials and methods, results, and discussion section. Nevertheless, in order to help the readers going throughout the work, each section is divided into two sub-sections presenting separately the studies on "Circulating" biomarkers from those on "ECG-derivided" biomarkers.

The following five studies were performed:

- ***CIRCULATING BIOMARKERS:***

- **STUDY 1: Discovery of new circulating biomarkers with the aid of untargeted metabolomics in a rat model of cardiac arrest**

BACKGROUND: The mechanisms responsible for post-cardiac arrest (CA) myocardial and cerebral dysfunction are not well understood, especially in the early post-resuscitation phases. In this investigation, we hypothesized that untargeted plasma metabolomic analyses would be a feasible approach to identify perturbations in circulating metabolites and thus potential mechanisms accounting for outcome of CA.

METHODS: Twelve rats, 450 ± 30 g, were used. Ventricular fibrillation (VF) was induced in 6 rats and was untreated for 6 min. Cardiopulmonary resuscitation (CPR), including mechanical chest compressions (CC), ventilations and epinephrine, was then initiated and continued for additional 6 min prior to defibrillation (DF). Two hr following resuscitation, animals were sacrificed and plasma collected. The other 6 rats were not subjected to CA and served as controls. We adopted unbiased mass spectrometry-based metabolomic profiling to identify perturbations in circulating metabolites. More specifically, plasma metabolome was examined by liquid chromatography-tandem mass spectrometry. Differences in chromatogram profiles were then analyzed using SIEVE v1.3 analysis software. Chromatographic peaks, with accurate masses, retention time and tandem mass fragmentation patterns were then identified using mass-spectral libraries of metabolites.

RESULTS: Our findings strongly indicated early alterations in a major route of the tryptophan (TRP) catabolism, namely the kynurenine pathway (KP), after resuscitation. Specific metabolites involved in the TRP catabolism were quantified absolutely using liquid chromatography-multiple reaction monitoring-mass spectrometry. TRP plasma concentration felt significantly in the early post-resuscitation phase, while its metabolites,

l-kynurenine (KYN), kynurenic acid (KYNA), and 3-hydroxyanthranilic acid (3-HAA) rose significantly ($p<0.05$).

CONCLUSIONS: Our results suggest that KP is activated early following resuscitation from CA. It is well known that KP is involved in the pathogenesis of numerous central nervous system disorders and hypotension during sepsis. Thus, KP activation might contribute the severity of post-CA syndrome.

- **STUDY 2: Validation of KP activation after cardiac arrest with experimental models in rats and pigs and in a small cohort of cardiac arrest patients**

BACKGROUND: KP is a major route of the TRP catabolism. In the present study, TRP and KP metabolites concentrations were measured in plasma from rats, pigs and humans after CA in order to assess KP activation and its potential role in post-resuscitation outcome.

METHODS: Plasma was obtained from: (A) 24 rats, subjected to 6 min CA and 6 min of CPR; (B) 10 pigs, subjected to 10 min CA and 5 min CPR; and (C) 3 healthy human volunteers and 5 patients resuscitated from CA. KP metabolites were quantified by liquid chromatography multiple reaction monitoring mass spectrometry. Assessments were available at baseline, and 1-4 hr, and 3-5 days post-CA.

RESULTS: KP was activated after CA in rats, pigs, and humans. Decreases in TRP occurred during the post-resuscitation period and were accompanied by significant increases in its major metabolites, 3-HAA and KYNA in each species, that persisted up to 3-5 days post-CA ($p<0.01$). In rats, changes in KP metabolites reflected changes in post-resuscitation myocardial function. In pigs, changes in TRP and increases in 3-HAA were significantly related to the severity of cerebral histopathogical injuries. In humans, KP activation was observed, together with systemic inflammation. Post-CA increases in 3-HAA were greater in patients that did not survive.

CONCLUSIONS: In this fully translational investigation, the KP was activated early following resuscitation from CA in rats, pigs, and humans, and might have contributed to post-resuscitation outcome.

- **STUDY 3: Validation of KP activation after cardiac arrest in a large cohort of out of hospital cardiac arrest patients**

BACKGROUND: KP is the major route of TRP catabolism and is activated by inflammation and after CA in animals. We have investigated post-CA KP activation in a large cohort of CA patients. We hypothesized that the KP activation level correlates with the severity of the ischemic insult, shock and long-term outcome.

METHODS: We performed a prospective multicentre observational study in 21 intensive care units (ICU) in Finland. Blood samples were obtained from 155 patients, for which prospective data collection included time to return of spontaneous circulation (ROSC), lowest systolic arterial pressure (SAP) and bicarbonate (BIC) during the first 24 hours of ICU care. Poor 12-month outcome was defined as a cerebral performance category (CPC) of 3-5. Plasma levels of TRP and its KP metabolites KYN, KYNA, 3-HAA, and the KYN/TRP ratio, were measured using liquid chromatography and mass spectrometry. Non-parametric tests, univariate, multivariate and linear regression analyses were used to determine associations of metabolites and study endpoints.

RESULTS: KP metabolites at ICU admission correlated with time to ROSC (KYN $p=0.005$, KYNA $p=0.023$, 3-HAA $p=0.011$, KYN/TRP $p=0.015$). Patients with higher levels of KYN, KYNA, and KYN/TRP ratio had lower 24 hr SAP and BIC. All KP metabolites, but not TRP, were significantly higher in patients with poor outcome (KYN, $p<0.001$, KYNA $p=0.043$, 3-HAA $p=0.047$, KYN/TRP $p<0.001$). Multivariable logistic regression showed that higher KYNA (OR 1.004, 95% CI 1.001-1.007, $p=0.038$) and 3-

HAA (OR 1.015, 95% CI 1.010-1.030, p=0.043) were independently associated with 12-month poor outcome.

CONCLUSIONS: KP is activated after CA and is associated with the severity of shock, early death and poor long-term neurological outcome. KP metabolites may have clinical value for prognostication.

- ***ECG-DERIVED BIOMARKERS:***

- **STUDY 4: AMSA evaluation in 609 VF patients in the United States**

BACKGROUND: The capability of Amplitude Spectrum Area (AMSA) to predict the success of DF was retrospectively evaluated in a large database of out-of-hospital CAs.

METHODS: Electrocardiographic data, including 1260 DFs, were obtained from 609 CA patients due to VF. AMSA sensitivity, specificity, accuracy, and positive and negative predictive values (PPV, NPV) for predicting DF success were calculated, together with receiver operating characteristic (ROC) curves. Successful DF was defined as the presence of spontaneous rhythm $\geq$ 40 bpm starting within 60 sec from the DF. In 303 patients with CC depth data collected with an accelerometer, changes in AMSA were analyzed in relationship to CC depth.

RESULTS: AMSA was significantly higher prior to a successful DF than prior to an unsuccessful DF (15.6±0.6 vs. 7.97±0.2 mV-Hz, $p<0.0001$). Intersection of sensitivity, specificity and accuracy curves identified a threshold AMSA of 10 mV-Hz to predict DF success with a balanced sensitivity, specificity and accuracy of almost 80%. Higher AMSA thresholds were associated with further increases in accuracy, specificity and PPV. AMSA of 17 mV-Hz predicted DF success in two third of instances (PPV of 67%). Low AMSA, instead, predicted unsuccessful DFs with high sensitivity and NPV >97%. Area under the

ROC curve was 0.84. CC depth affected AMSA value. When depth was <1.75 in, AMSA decreased for consecutive DFs, while it increased when the depth was >1.75 in ($p<0.05$).

CONCLUSIONS: AMSA could be a useful tool to guide CPR interventions and predict the optimal timing of DF.

- **STUDY 5: AMSA evaluation in 1.617 VF patients in Lombardia Region, Italy**

BACKGROUND: In a highly populated area of northern Italy, we evaluated the capability of AMSA to predict DF outcome in of out-of-hospital CA patients, concurrently to other ECG-derived parameters. We hypothesized that AMSA would be a good predictor of DF outcome and that threshold values of AMSA could be identified such to be used as a guide for CPR intervention, i.e. CC or DF.

METHODS: ECG data recorded by automated external defibrillators from different manufactures were obtained from cardiac arrest events occurring in 9 cities in Lombardia Region, Italy. Among these events, only VF CAs receiving DFs were selected. A database including 2.442 DFs, obtained from 1.050 patients enrolled between 2008-2009, was used as derivation group. An additional database, including 1.386 DFs, obtained from 567 patients enrolled in 2010, was used as validation group. A 2 sec ECG window ending at 0.5 sec before DF was analyzed and AMSA was calculated, together with root mean square (RMS), peak frequency (PF), median frequency (MDF), and mean frequency (MNF). Parametric and non-parametric tests, univariate, multivariate, linear regression analyses and area under the receiver operating characteristic (ROC) curve were used to determine associations of AMSA and ECG-parameters and study endpoints, i.e. DF success, ROSC, survival till hospital discharge, 6 months and 1 year. Threshold values of AMSA able to discriminate among successful and not successful DFs were individuated in the derivation database and sensitivity, specificity, positive and negative predictive values (PPV, NPV) were evaluated in the validation database.

RESULTS: Among the 2.447 DF attempts, 26.2% were successful. AMSA was significantly higher prior to a successful DF than a failing one (13±5 vs. 6.8±3.5, $p<0.0001$). RMS, PF, MDF, but not MNF, were also significantly higher prior to a successful DF compared to an unsuccessful one. AMSA was an independent predictor of DF outcome (OR 1.40, 95%CI 1.36-1.44) and ROSC (OR 1.21, 95%CI 1.16-1.25). Age, gender, EMS arrival, myocardial infarction, congestive heart failure, diabetes, and anti-hypertensive drug, were the factors associated with AMSA values. RMS, PF, MDF, but not MNF, also predicted DF outcome and ROSC. Nevertheless, area under the ROC curve for DF outcome prediction was significantly greater for AMSA compared to the other ECG-derived parameters (0.86, $p<0.0001$). Unexpectedly, AMSA was also associated with hospital discharge and long term survival. Intersection of sensitivity, specificity and accuracy curves identified a threshold value of AMSA of 8.9 mV-Hz, able to predict DF outcome, with a balanced sensitivity, specificity and accuracy of 79%. Moreover, intersection of PPV and accuracy curves identified a threshold value of AMSA of 15.5 mV-Hz able to predict a successful DF with a PPV and accuracy of 79%. AMSA values greater than 23 mV-Hz correctly predicted the success of DF with a PPV value of 100%. AMSA below 6.5 mV-Hz correctly predicted the DF failure with a NPV of > 95%. In the validation database, the AMSA threshold of 15.5 predicted DF success with a PPV > 80% and a specificity of 97%, while the AMSA threshold of 6.5 predicted DF failure with a NPV > 98% and a sensitivity > 97%.

CONCLUSIONS: In this population, one of the largest studied to now, AMSA was a better predictor of DF outcome compared to other ECG-derived parameters. Indeed, AMSA was confirmed to predict DF outcome with high accuracy. A specific AMSA threshold in order to predict DF outcome, i.e. success or failure, may be identified during CPR. An AMSA-based DF decision therefore would be an useful approach to guide the best CPR intervention.

INTRODUCTION

Cardiac arrest represents a dramatic clinical event that can occur suddenly and often without premonitory signs. This condition is characterized by sudden loss of consciousness caused by the lack of cerebral blood flow, which occurs when the heart ceases to pump. Indeed, it represents a leading cause of death in the western world, with as many as 350.000 to 700.000 people in the United States, Canada and Europe sustaining cardiac arrest each year *(Lippert 2010, Nolan 2010, Travers 2010)*. Cardiopulmonary resuscitation (CPR), including chest compression (CC), often in conjunction with electrical defibrillation (DF), has the potential of re-establishing spontaneous circulation. Despite major efforts to improve outcomes from cardiac arrest, average survival rate remains dismal and presents a large variation with a spread between 2 to 50% *(Atwood 2005, Eisenberg 2009, Fredriksson 2003, Nichol 2008, Travers 2010)*. Though the initial success of CPR, in fact, the majority of victims die within 72 hr from hospital admission, due what is now called "post cardiac arrest syndrome" *(Brown 1992, Nolan 2008, Peberdy 2010, Sasson 2010, Schenenberger 1994)*. Most prominent are post resuscitation myocardial failure, ischemic brain damage and processes related to the systemic ischemia/reperfusion response *(Nolan 2008)*. Severe heart contractile failure has been implicated as one of the most important mechanism accounting for the early fatal outcome *(Gazmuri 2012, Laurent 2002, Tang 1993, van Alem 2003)*. Long term morbidity and mortality after successful CPR, instead, largely depend on recovery of neurologic function (*Nolan 2008, Sandroni 2013 and 2013b)*. As many as 30% of survivors of cardiac arrest, in fact, manifest permanent brain damage and in some instances only 2–12% of resuscitated patients have been discharged from the hospital without neurological impairments *(Böttiger 1999, Brain Resuscitation Clinical Trial I 1986, Brown 1992, Nolan 2008, Olasveengen 2009, Peberdy 2010, Schenenberger 1994)*.

The mechanisms responsible for post-cardiac arrest myocardial and cerebral injury are not well understood, although several events have been described. The reintroduction of oxygenated blood after the return of spontaneous circulation (ROSC) stimulates a sequence

of complex actions that lead to acute inflammatory responses and release of reactive oxygen species (ROS), causing oxidative damage, cellular edema, cell membrane damage, calcium ion (Ca^{2+}) overload, mitochondrial dysfunction, and apoptosis *(Dezfulian 2007, Lebuffe 2003, Levraut 2003, Nolan 2008, Ouyang 1999, Polderman 2009)*. Several other processes, including interactions between pleiotropic mediators, coagulation abnormalities, activation of the inflammatory cytokine cascade, chemokine upregulation and ultimately recruitment of inflammatory leukocytes and reactive astrogliosis have also been reported after cardiac arrest and are major players in the final outcome *(Adrie 2002 and 2004 and 2005, Frangogiannis 1998, Meybohm 2009, Neumar 2008, Nolan 2008, Vakeva 1998)*.

There is still controversy about how much the clinical use of current biomarkers, i.e. cardiac troponins (cTn), neuron-specific enolase (NSE), protein S-100 beta (S-100b) contributes to the prediction of outcome of cardiac arrest *(Nolan 2008, Peberdy 2010, Scolletta 2012)*. Predicting survival, myocardial and neurological outcome is therefore a difficult issue, especially in the early post-resuscitation phase.

CPR and defibrillation

Ventricular fibrillation (VF) is one of the primary rhythms in many cases of cardiac arrest. Indeed, on initial heart rhythm analysis, about 25–30% of out of hospital cardiac arrests are due to VF *(Agarwal 2009, Cobb 2002, Nolan 2010, Rea 2004, Ringh 2009, Travers 2010, Vaillancourt 2007)*. It is likely that many more victims have VF or rapid ventricular tachycardia (VT) at the time of collapse but, by the time the first electrocardiogram (ECG) is recorded the rhythm has deteriorated to asystole. When the rhythm is recorded soon after collapse, in particular by an on-site automated external defibrillator (AED), the proportion of patients in VF can be as high as 65% *(Nolan 2010, Travers 2010)*. DF by electrical counter-shock represents the treatment of choice for this otherwise lethal arrhythmia. Electrical DF refers to the passage across the myocardium of an electrical current of sufficient magnitude to depolarize a critical mass of myocardium and to enable restoration of coordinated electrical

activity. Indeed, successful DF is defined as the *termination of fibrillation* or, more precisely, the absence of VF/ventricular tachycardia (VF/VT) within 5 sec after the shock delivery; however, the goal of attempted DF is to restore spontaneous circulation *(Deakin 2005 and 2010, Link 2010)*. The probability of successful DF diminishes rapidly over time. For every min that passes between collapse and DF, survival rates from witnessed VF cardiac arrest decrease by 7% to 10% if no CPR is provided. When bystander CPR is provided, the decrease in survival rates is more gradual and averages 3% to 4% per min from collapse to DF attempt *(Deakin 2005, Larsen 2003, Link 2010, Valenzuela 1997, Waalewijn 2001)*. VF is, in fact, characterized by three time-sensitive electrophysiological phases, including 1) the electrical phase of 0-4 min, 2) the circulatory phase of 4-10 min and 3) the metabolic phase of > 10 min. During the electrical phase, immediate DF is likely to be successful. As ischemia progresses, the success of attempted DF diminishes without CPR. This phase is characterized by transition to slow VF wavelets during accumulation of ischemic metabolites in the myocardium. Slow VF is often resistant to DF because there is no longer an excitable gap to interrupt the re-entry that sustains VF. The failure of DF to succeed in slow VF can be attributed to re-entry and recurrence of VF. In the metabolic phase, there is much less likelihood of successful ROSC *(Weisfeldt 2002)*.

During cardiac arrest, coronary blood flow ceases, accounting for a progressive and severe energy imbalance. Intra-myocardial hypercarbic acidosis is associated with depletion of high energy phosphates and correspondingly severe global myocardial ischemia *(Johnson 1995, Kern 1990)*. The ischemic left ventricle (LV) becomes contracted ushering in the "stone heart" **(Figure 1)** *(Klouche 2000 and 2002)*. After the onset of contracture, the probability of successful DF becomes remote.

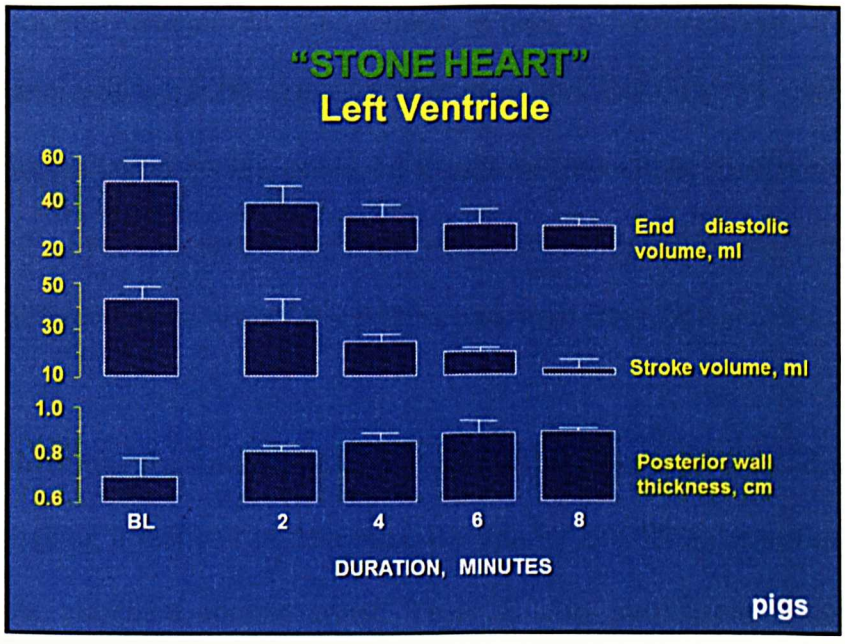


Figure 1.
Development of the stone heart with reduced LV end-diastolic volume, stroke volume and increased LV wall thickness over 8 min of cardiac arrest in pigs (modified from *Klouche 2002*).

Early CPR, with partial restoration of coronary perfusion pressure (CPP) and myocardial blood flow, delays onset of ischemic myocardial injury and facilitates DF *(Deshmukh 1989)*. Accordingly, major factors contributing to the poor outcome after cardiac arrest include delays in CPR, ineffective and frequently interrupted CC, and limited access to, or delayed DF *(Iwami 2012, Valenzuela 2000, Wik 2005)*.

Indeed, timing of DF in relationship to CC has been a subject of major interest. Based on available evidence, the 2005 guidelines recommended an initial interval of CC prior to DF, especially when the duration of untreated cardiac arrest exceeded 4 min *(Wik 2005, AHA guidelines 2005)*. Nevertheless, the recent 2010 guidelines highlighted the insufficient evidence to support or refute CPR before DF and called again for early DF *(Baker 2008, Deakin 2010, Jacobs 2005, Link 2010)*. Subsequent DF has been recommended to be attempted on a time based protocol, i.e. after every 2 min cycle of CC *(Deakin 2010, Link 2010)*, which may lead to futile DF attempts and unnecessary CC interruptions, potentially creating worse outcome *(Cheskes 2011, Xie 1997, Yu 2002, Snyder 2004, Steen 2003)*. The timing of DF is even more difficult to be chosen in the instance of recurrence of VF *(Shanmugasundaram 2012)*. In addition, the severity of post resuscitation myocardial dysfunction and survival

has been recognized to be related, in part, to the magnitude of the total electrical energy delivered with DF *(Osswald 1994, Tang 2004 and 2006, Xie 1997)*. Indeed, increasing the defibrillation energy produced significant reductions in survival rate, cardiac index, and LV function. Better survival and post resuscitation myocardial function have been observed when lower DF energies were used *(Tang 1999 and 2004, Xie 1997)*.

However, the onset time of VF is rarely known, especially in the out-of-hospital setting, making it difficult to determine the priority of CPR intervention based on the duration of the untreated cardiac arrest. There is also insufficient knowledge about the optimal duration of the CC interval prior to DF. The decision whether to interrupt CC to deliver a DF is therefore difficult. ECG analysis of the VF waveform might represent the best non-invasive decision guide *(Callaway 2005)*.

Monitoring effectiveness of CC and predicting DF success

The evidence is secure that the quality of CC is a major determinant of successful resuscitation *(Abella 2005, Gallagher 1995, Idris 2012, Vaillancourt 2011, Van Hoeyweghen 1993, Wik 1994 and 2005)*. Existing and established predictors of good quality CPR and thereby successful resuscitation include CPP *(Deshmukh 1989, Paradis 1990, Sanders 1985 and 1985b, Yu 2002)*, and end-tidal CO_2 ($EtCO_2$) *(Falk 1988, Lah 2011, Headstveit 2012, Kolar 2008, Weil 1985)*.

Blood flows generated by CC are dependent on the pressure gradient between the aortic and the venous pressures *(Andreka 2006)*. CPP, defined as the difference between simultaneously measured minimal aortic pressure and right atrial pressure during compression diastole *(Kette 1991, Gazmuri 1996, Tang 1993 and 1999)*, is highly correlated with coronary blood flow during cardiac resuscitation and is currently recognized as the best single indicator of the likelihood of successful DF and ROSC *(Niemann 1985, Paradis 1990, Povoas 2000)*. Based on both experimental and clinical observations, ROSC can be predicted

when CPP is maintained above 15 mmHg during CC *(Kern 1988, Niemann 1985. Paradis 1990)*. Resuscitative strategies that increase CPP, including high quality CC *(Ristagno 2007, Wik 1996)* as well as the use of vasopressors *(Lewis 1969, Mentzelopoulos 2013, Olasveengen 2012)*, have been therefore supported and considered more effective in restoring spontaneous circulation.

Expired CO_2 is determined by the body's production of CO_2 and the relationship between minute ventilation and pulmonary perfusion. When the circulatory status is normal, pulmonary perfusion is in the physiologic ranges and $EtCO_2$ is determined by minute ventilation. Under condition of cardiac arrest and CPR, the cardiac output (CO) is usually less than one-third of normal and pulmonary flow and $EtCO_2$ are consequently dramatically reduced. $EtCO_2$ is therefore an indirect measurement of pulmonary blood flow and CO produced by CCs *(Falk 1988, Weil 1985)*. End-tidal CO_2 is highly correlated with CPP during CPR, and may thereby serve as a non-invasive surrogate of CPP *(Gudipati 1988, von Planta 1989)*. EtCO2 has emerged as another valuable tool for monitoring the effectiveness of CCs during CPR *(Falk 1988, Garnett 1987, Neumar 2010, Ristagno 2007, Weil 1985)*. When $EtCO_2$ exceeds the threshold level of approximately 10 to 15 mmHg during CPR, greater likelihood of successful ROSC has been reported *(Cantineau 1996, Grmec 2001, Neumar 2010)*. In addition, $EtCO_2$ may also provide the earliest clinical evidence of ROSC *(Neumar 2010, Falk 1988)*. However, this measurement has not been yet specifically evaluated in the setting of prediction of DF success in humans.

Although the importance of blood pressure during CPR is clear, invasive measurements, including aortic and right atrial pressures are only available or feasible at the time of resuscitation in a small minority of patients, especially in the pre-hospital settings. Similarly, $EtCO_2$, a good readout of the effectiveness of CC and therefore of the likelihood of ROSC *(Chase 1993, Gudipati 1988, Kern 1989, Neumar 2010, Weil 1985)*, is not widely available

yet. Portable infrared capnometers can be successfully employed in pre-hospital settings during CPR; however, monitoring $EtCO_2$ requires airway adjuncts. Out of hospital endotracheal intubation carries a high failure rate and a 30% incidence of traumatic injury to the airway *(Domino 1999, Köhler 2008)*. Moreover administration of epinephrine during CPR causes significant decreases in $EtCO_2$ by increasing pulmonary shunting *(Tang 1991)*. This therefore might cause important mis-interpretation when $EtCO_2$ is employed to monitor and guide the resuscitative maneuvers *(Gonzalez 1989)*.

These restraints in the use of CPP and $EtCO_2$ are in contrast with the routine availability of the ECG in current external defibrillators. The attention, with the intent to identify a better predictor of ROSC, has been therefore focused on the analyses of electrocardiographic features of VF waveform. The development of a non-invasive and real time monitoring that allowed prediction of whether or not a shock would cause ROSC is therefore of great importance. Current methods are constrained in part by the lack of practical and reliable real-time tools for monitoring the efficacy of CPR interventions and for guiding the appropriate timing for defibrillation attempts. At present, the electrocardiographic analyses of VF waveform might represent the best non-invasive approach to guide the priority of interventions, namely CC or DF.

Post-cardiac arrest syndrome

After the initial success of CPR, the majority of resuscitated patients die within 72 hr, due to what is now termed "post cardiac arrest syndrome" *(Lippert 2010, Nolan 2008, Travers 2010)*. Indeed, ROSC after cardiac arrest is an unnatural pathophysiological state created by successful CPR. In the early 1970s, Dr. Vladimir Negovsky recognized that the pathology caused by complete, whole-body ischemia and reperfusion was unique in that it had a clearly definable cause, time course, and constellation of pathophysiological processes *(Negovsky 1972 and 1988 and 1995)*. Negovsky named this state "post resuscitation disease".

Although appropriate at the time, the term "resuscitation" is now used more broadly to include treatment of various shock states in which circulation has not ceased. Moreover, the term "post resuscitation" implies that the act of resuscitation has ended. Negovsky stated that "a second, more complex phase of resuscitation begins when patients regain spontaneous circulation after cardiac arrest" *(Negovsky 1972)*. Therefore, the term "post-cardiac arrest syndrome" seems more appropriate. The 3 key components of this syndrome are: 1) post cardiac arrest brain injury; 2) post cardiac arrest myocardial dysfunction; 3) systemic ischemia/reperfusion response. This state is often complicated by a fourth component: the unresolved pathological process that caused the cardiac arrest *(Neumar 2008, Nolan 2008)*. Pathophysiology, clinical manifestations and potential treatments of the post cardiac arrest syndrome are summarized in **Table 1.**

Table 1. Post-cardiac arrest syndrome: pathophysiology, clinical manifestations, and potential treatments.

Syndrome	Pathophysiology	Clinical manifestation	Potential treatments
Post-cardiac arrest brain injury	• Impaired cerebrovascular autoregulation • Cerebral oedema (limited) • Postischaemic neurodegeneration	• Coma • Seizures • Myoclonus • Cognitive dysfunction • Persistent vegetative state • Secondary Parkinsonism • Cortical stroke • Spinal stroke • Brain death	• Therapeutic hypothermia • Early haemodynamic optimization • Airway protection and mechanical ventilation • Seizure control • Controlled reoxygenation (SaO_2 94%-96%) • Supportive care
Post-cardiac arrest myocardial dysfunction	• Global hypokinesis (myocardial stunning) • Reduced cardiac output • ACS	• Early revascularization of AMI • Hypotension • Dysrhythmias • Cardiovascular collapse	• Early haemodynamic optimization • Intravenous fluid • Inotropes • IABP • LVAD • ECMO
Systemic ischaemia/ reperfusion response	• Systemic inflammatory response syndrome • Impaired vasoregulation • Increased coagulation • Adrenal suppression • Impaired tissue oxygen delivery and utilisation • Impaired resistance to infection	• Ongoing tissue hypoxia/ischaemia • Hypotension • Cardiovascular collapse • Pyrexia (fever) • Hyperglycaemia • Multiorgan failure • Infection	• Early haemodynamic optimization • Intravenous fluid • Vasopressors • High-volume haemofiltration • Temperature control • Glucose control • Antibiotics for documented infection
Persistent precipitating pathology	• Cardiovascular disease (AMI/ACS, cardiomyopathy) • Pulmonary disease (COPD, asthma) • CNS disease (CVA) • Thromoboembolic disease (PE) • Toxicologic (overdose, poisoning) • Infection (sepsis, pneumonia) • Hypovolaemia (haemorrhage, dehydration)	• Specific to aetiology, but complicated by concomitant PCAS	• Disease-specific interventions guided by patient condition concomitant PCAS

ACS indicates acute coronary syndrome; AMI, acute myocardial infarction; IABP, intra-aortic balloon pump; LVAD, left ventricular assist device; EMCO, extracorporeal membrane oxygenation; COPD, chronic obstructive pulmonary disease; CNS, central nervous system; CVA, cerebrovascular accident PE, pulmonary embolism; and PCAS, post-cardiac arrest syndrome.

From *Nolan 2008*

A more treatment-orientated approach would be to define the phases of post-cardiac arrest syndrome by time (**Figure 2**). Indeed, 4 phases are recognized: 1. the *immediate* post-arrest phase, including the first 20 min after ROSC; 2. the *early* post-arrest phase, defined as the period between 20 min and 6-12 hr after ROSC, when early interventions might be most effective; 3. The *intermediate*, between 6-12 and 72 hr, when injury pathways are still active and aggressive treatment is typically instituted; and 4. the period beyond 3 days after ROSC, considered the *recovery* phase when prognostication becomes more reliable and ultimate outcomes are more predictable.

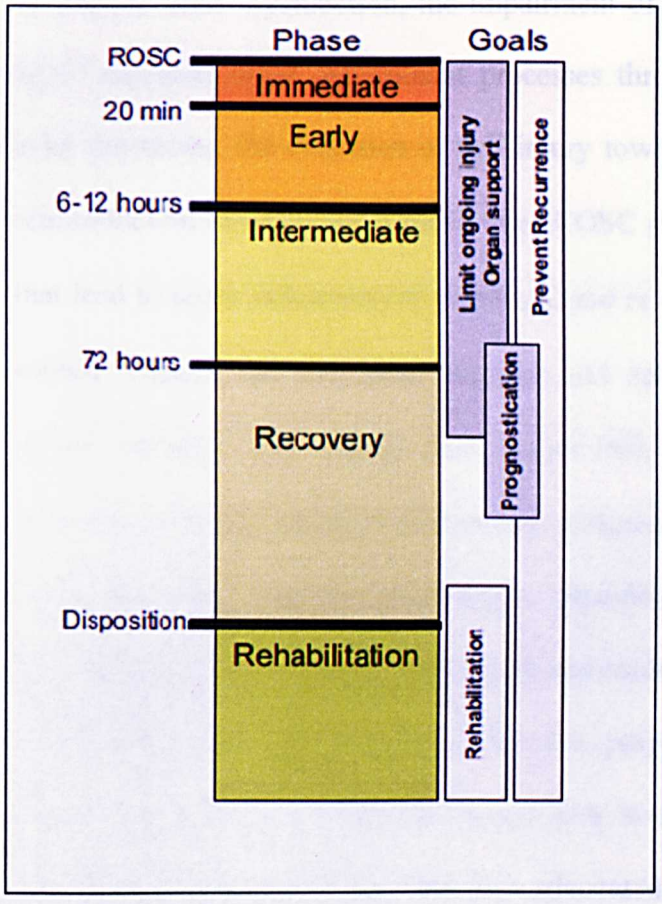


Figure 2. Temporal phases of the post-cardiac arrest syndrome. From *Nolan 2008*.

Within the first 24 hr post-arrest, a microcirculatory dysfunction from the multifocal ischemia/reperfusion leads to rapid release of toxic enzymes and free radicals into the cerebrospinal fluid and blood. Over the next 1-3 days, cardiac and systemic functions improve, but intestinal permeability increases, predisposing the patient to sepsis and the multiple organ dysfunction syndrome. During the subsequent days, a serious infection may occur causing rapid clinical deterioration. The patient either dies of a complication or of

the primary disease that caused the cardiac arrest, or undergoes a partial or complete recovery.

The mechanisms responsible for post-cardiac arrest myocardial and cerebral injury are not well understood, although several events have been described. The condition of systemic ischemia that follows onset of cardiac arrest may lead to processes of metabolism breakdown and subsequent primary cell necrosis *(Radovsky 1995)*. In addition, the profound imbalance between adenosine triphosphate (ATP) synthesis and utilization, a consequence of mitochondrial dysfunction, the impairment of ionic homeostasis and the formation of ROS, represent other determinant processes through which mitochondria accelerate, or even determine, the evolution of cell injury toward necrosis or apoptosis. Moreover, the reintroduction of oxygenated blood after ROSC stimulates a sequence of complex actions that lead to acute inflammatory responses and release of ROS, causing oxidative damage, cellular edema, cell membrane damage and apoptosis, collectively called "reperfusion injury" *(Dezfulian 2007, Lebuffe 2003, Levraut 2003, Nakka 2008, Ouyang 1999)*. Several other processes, including interactions between pleiotropic mediators, coagulation abnormalities, activation of the inflammatory cytokine cascade, chemokine upregulation and ultimately recruitment of inflammatory leukocytes and reactive astrogliosis have been also reported after cardiac arrest and have been shown to play major roles in the determination of the final outcome *(Adrie 2002 and 2005, Neumar 2008, Nolan 2008, Lebuffe 2003, Ouyang 1999)*. Release of excitatory aminoacids has been also advocated as another pathway leading to neuronal damage *(Neumar 2000 and 2008, Polderman 2009)*.

Post-cardiac arrest brain injury

The unique vulnerability of the brain is attributed to its limited tolerance of ischemia as well as its unique response to reperfusion. The mechanisms of brain injury triggered by cardiac arrest and resuscitation are complex and include excitotoxicity, disrupted Ca^{2+}

homeostasis, free radical formation, pathological protease cascades, and activation of cell death signaling pathways *(Lipton 1999, Neumar 2000 and 2008, Nolan 2008, Polderman 2009)*. Both neuronal necrosis and apoptosis have been reported after cardiac arrest. Histologically, selectively vulnerable neuron subpopulations in the hippocampus, cortex, cerebellum, corpus striatum, and thalamus, degenerate over hours to days *(Brierley 1973, Blomqvist 1985, Hossmann 2001, Neumar 2000, Nolan 2008, Polderman 2009, Pulsinelli 1985, Taraszewska 2002)*. Levels of high energy metabolites such as ATP and phosphocreatine decrease within seconds when oxygen supply to the brain is interrupted *(Small 1999)*. The breakdown of ATP and the switch of intracellular metabolism to anaerobic glycolysis lead to an increase in intracellular levels of inorganic phosphate, lactate, and hydrogen ion, resulting in both intra- and extracellular acidosis (**Figure 3**).

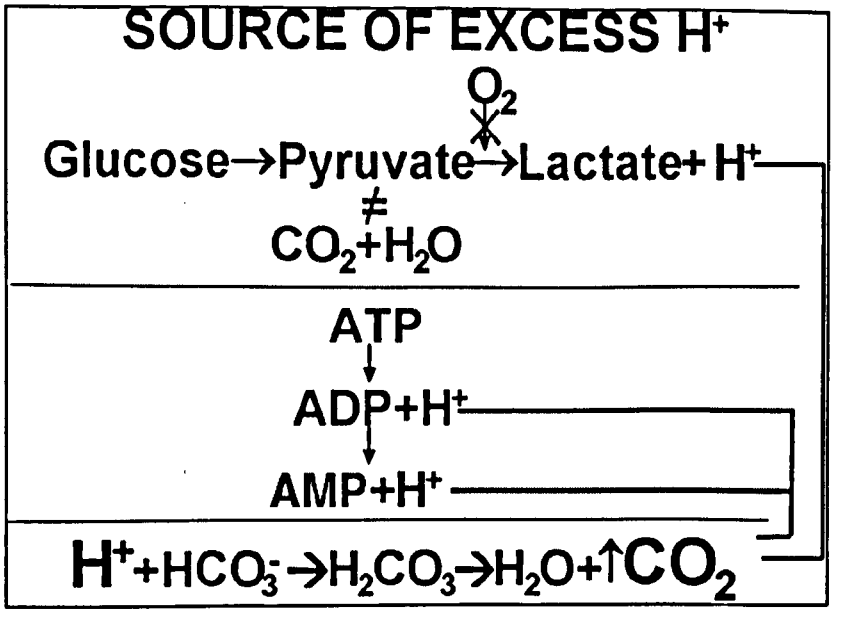


Figure 3. Source of hydrogen ions (H^+) during low-flow states. H^+ derived from the anaerobic oxidation of glucose and from the hydrolysis of high-energy phosphates. ATP, adenosine 5' triphosphate; ADP, adenosine 5' diphosphate; AMP, adenosine monophosphate.
From *Ristagno 2006*.

The above processes lead to influx of Ca^{2+} into the cell. Loss of ATP and acidosis also inhibit the mechanisms that normally deal with excessive intracellular Ca^{2+} by sequestering Ca^{2+} from the cell, further aggravating intracellular Ca^{2+} overload. These problems are compounded by failure of ATP–dependent (sodium-potassium) Na^+-K^+ pumps and K^+, Na^+, and Ca^{2+} channels, leading to an additional influx of Ca^{2+}. The excess Ca^{2+} induces

mitochondrial dysfunction (increasing intracellular Ca^{2+} influx yet further, in a vicious cycle) and activates numerous intracellular enzyme systems (kinases and proteases). In addition, immediate early genes are activated and a depolarization of neuronal cell membranes occurs, with a release of large amounts of the excitatory neurotransmitter glutamate into the extracellular space *(Neumar 2000, Polderman 2009, Siesjo 1989)*. This leads to prolonged and excessive activation of membrane glutamate receptors, further stimulating Ca^{2+} influx through activation of Ca^{2+} channels in another vicious cycle. Under normal circumstances, neurons are exposed to only very brief pulses of glutamate; prolonged glutamate exposure induces a permanent state of hyperexcitability in the neurons (the *excitotoxic cascade*), which can lead to additional injury and cell death. A destructive process that is closely linked to but distinct from the mechanisms discussed above is the release of ROS following ischemia/reperfusion. Mediators such as superoxide, peroxynitrite, hydrogen peroxide, and hydroxyl radicals play an important role in determining whether injured cells will recover or die *(Globus 1995 and 1995b, Novack 1996, Polderman 2009)*. Free radicals can oxidize and damage numerous cellular components. Although brain cells have various enzymatic and non-enzymatic antioxidant mechanisms that prevent this type of injury under normal circumstances, ROS production following ischemia/reperfusion is so great that these defensive mechanisms are likely to be overwhelmed, leading to peroxidation of lipids, proteins, and nucleic acids. Ischemia/reperfusion can also lead to significant disruptions in the blood–brain barrier (BBB), which can facilitate the subsequent development of brain edema *(Chi 2001, Huang 1999)*. All the above processes contribute to the development of post-cardiac arrest intracranial hypertension, which further augments brain damage (**Figure 4**).

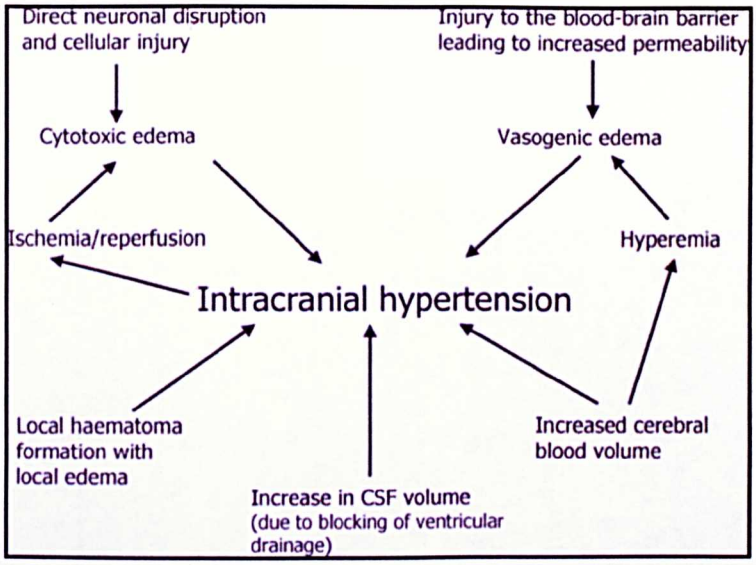


Figure 4. Schematic depiction of the potential role of intracranial pressure as both a marker of ongoing brain injury and a potential cause of additional injury, and of the factors that may contribute to a rise in intracranial pressure.
CSF, cerebrospinal fluid.
From *Polderman 2009.*

Mechanisms accounting for BBB loss of integrity include: decreased fluidity and integrity of cell membranes and increased vascular permeability of microvascular endothelial cells in the brain, mediated by inflammatory cytokines and vascular endothelial growth factor *(Kaur 2008)*, *via* release of nitric oxide (NO) *(Fischer 1999)*. More specifically NO can interact with ROS producing peroxynitrite radicals, which activate matrix metalloproteinases (MMPs) that ultimately disrupt BBB junctions (**Figure 5**).

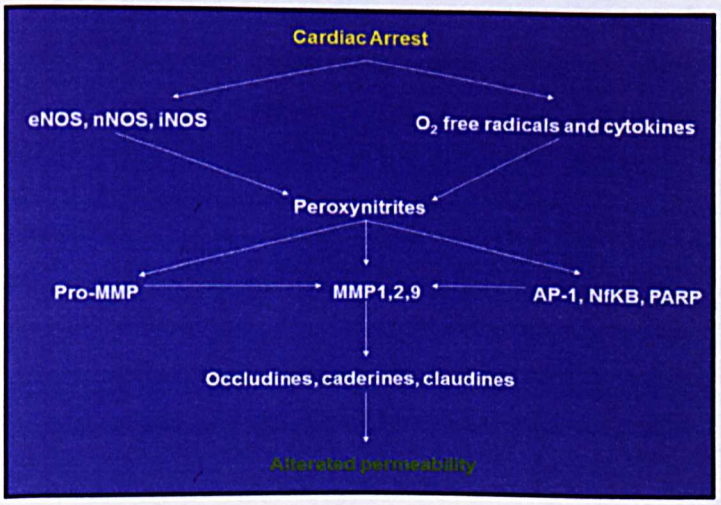


Figure 5. Schematic depiction of mechanisms involved in BBB disruption. e- endothelial, n- neuronal, i- inducible, nitric oxide synthase (NOS); Matrix metalloproteinases (MMP); the activator protein 1 (AP-1); nuclear factor kappa beta (NfKB); Poly ADP-ribose polymerase (PARP).

Prolonged cardiac arrest can also be followed by fixed and/or dynamic failure of cerebral microcirculatory reperfusion despite adequate cerebral perfusion pressure *(Fischer 1996)*.

This impaired reflow can cause persistent ischemia and small infarctions in some brain regions. The cerebral microvascular occlusion that causes no-reflow has been attributed to intravascular thrombosis during cardiac arrest and has been shown to be responsive to thrombolytic therapy in preclinical studies. Cardiopulmonary arrest and resuscitation are accompanied by a marked activation of coagulation, which can lead to intravascular fibrin formation with blockage of the microcirculation (**Figure 6**). Other phenomena may be involved in cerebral perfusion disturbances, that include not only no-reflow events but also hyperemia episodes following ischemia: increased blood viscosity and perivascular edema, as well as possible down-regulation of NO synthesis, expression of endothelial adhesion molecules and generation of free radicals *(Bottiger 1997, Donadello 2011, Hossmann 1995, Liachenko 2001, van Genderen 2012)*.

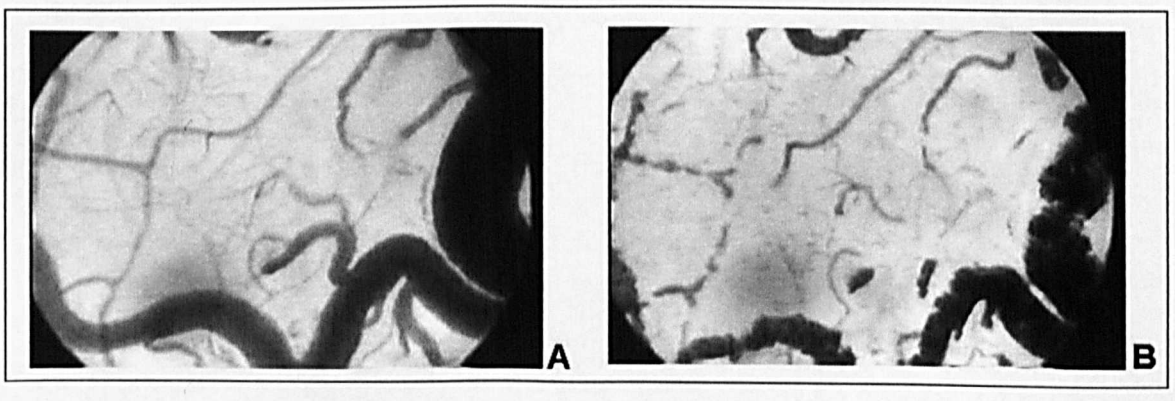


Figure 6. Digital photomicrographs of the cerebral cortical circulation at (A) baseline before induction of cardiac arrest; and (B) 2 min after onset of cardiac arrest. From *Ristagno 2008.*

Beyond the initial reperfusion phase, several factors can potentially compromise cerebral oxygen delivery and possibly secondary injury in the hours to days after cardiac arrest. These include hypotension, hypoxemia, impaired cerebrovascular autoregulation, and brain edema. Furthermore, hyperglycemia is common in post-cardiac arrest patients and is importantly associated with poor neurological outcome after cardiac arrest *(Calle 1989, Longstreth 1983 and 1984 and 1993, Mullner 1997, Skrifvars 2003)*.

Post-cardiac arrest myocardial dysfunction

Post-cardiac arrest myocardial dysfunction contributes to the early deaths after resuscitation from cardiac arrest *(Herlitz 1995, Laurent 2002, Lavert 2004, Neumar 2008, Nolan 2008)*. Laboratory and clinical evidence, however, indicates that this phenomenon is both responsive to therapy and reversible *(Cerchiari 1993, Huang 2005, Kern 1996 and 1997, Laurent 2002, Nolan 2008, Ruiz-Bailén 2005)*. Immediately after ROSC, heart rate (HR) and blood pressure are extremely variable and this is mainly caused by a transient increase in myocardial and circulating catecholamine concentrations *(Prengel 1992, Rivers 1994)*. Premature ventricular beats and episodes of VT and VF commonly occur during the early minutes after resuscitation and account for early death. Furthermore, an overall condition of severe myocardial dysfunction, including variable degrees of systolic and diastolic dysfunction, is present. During this period with significant myocardial dysfunction, coronary blood flow however is not reduced, indicating a true *stunning* phenomenon rather than a permanent injury or infarction (**Figure 7**).

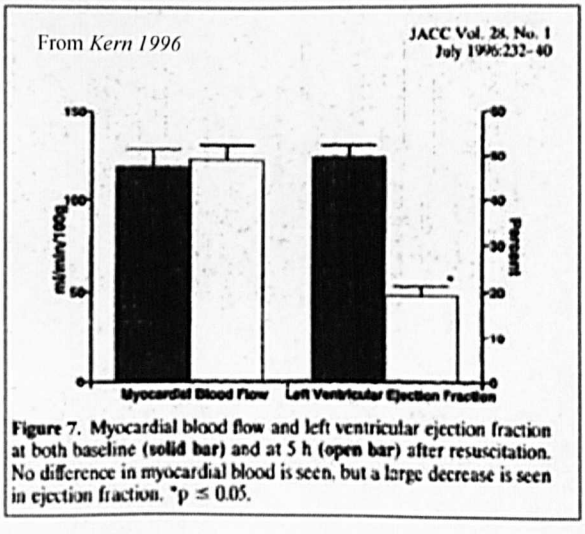


Figure 7. Myocardial blood flow and left ventricular ejection fraction at both baseline (**solid bar**) and at 5 h (**open bar**) after resuscitation. No difference in myocardial blood is seen, but a large decrease is seen in ejection fraction. *$p \leq 0.05$.

In one series of 148 patients who underwent coronary angiography after cardiac arrest, 49% of subjects had myocardial dysfunction manifested by tachycardia and elevated LV end-diastolic pressure, followed approximately 6 hr later by hypotension and low CO *(Kern 1996, Laurent 2002)*. This global dysfunction was transient, and full recovery occurred. In a swine model with no antecedent coronary or other LV dysfunction features, the time to

recovery appeared to range between 24 and 48 hr. Several case series have described transient myocardial dysfunction after human cardiac arrest. Cardiac index values reached their nadir at 8 hr after resuscitation, improved substantially by 24 hr, and almost uniformly returned to normal by 72 hr in patients who survived out-of-hospital cardiac arrest. This trend in arterial pressure, cardiac index and other hemodynamic parameters are described in the **Table 2** from Dr. Laurent's work *(Laurent 2002)*, where clinical data over the 72 hr post cardiac arrest are reported.

Hemodynamic Data During the First 72 Hours — **Table 2** (From *Laurent 2002*)

Hemodynamic Parameters	Interval From Onset of Cardiac Arrest to Measurement (h)						
	ICU Admission: 3.0 (2.0–3.6)	Time 0: 6.8 (4.3–7.3)	Time 1: 8.0 (7.0–9.0)	Time 2: 12.0 (11.0–13.5)	Time 3: 24.0 (23.0–25.7)	Time 4: 67.0 (52.0–72.0)	p Value§
Temperature (°C)	36.0 (35.4–36.7)	—	36.6* (35.8–37.5)	37.3† (36.7–38.1)	37.6† (37.0–38.2)	37.8† (37.3–38.3)	<0.001
Epinephrine perfusion (mg/h)	0	0	1.0‡ (0–2.2)	1.3‡ (0–2.0)	1.5‡ (0–2.7)	0.4‡ (0–1.6)	0.042
HR (beats/min)	110 (89–123)	111 (91–124)	108 (97–125)	111 (98–128)	112 (101–125)	101* (94–120)	0.215
MAP (mm Hg)	87 (75–103)	62† (46–71)	79‡ (69–102)	76‡ (69–87)	80 (71–89)	80 (73–88)	0.04
MPAP (mm Hg)	—	—	28 (22–32)	24‡ (20–28)	24† (20–27)	28 (24–32)	0.005
RAP (mm Hg)	—	—	11 (8–15)	10 (8–13)	11 (8–13)	12 (9–15)	0.189
POAP (mm Hg)	—	—	14 (11–18)	12‡ (10–15)	13 (10–16)	14 (10–18)	0.248
CI (l/min per m^2)	—	—	2.05 (1.43–2.90)	2.61‡ (1.90–3.46)	3.19‡ (2.67–4.20)	3.69* (2.92–4.49)	<0.001
SVRI (dynes·s/cm^5·m^2)	—	—	2,908 (1,946–4,658)	1,936† (1,493–2,951)	1,672† (1,300–2,034)	1,518† (1,153–1,852)	<0.001
PVRI (dynes·s/cm^5·m^2)		—	438 (339–593)	363† (221–488)	261† (183–346)	274* (206–371)	<0.001
SI (ml/m^2)		—	20.0 (15.0–23.8)	22.5‡ (18.4–32.1)	29.3† (24.8–37.4)	35.3‡ (28.5–42.1)	<0.001
LVSW (g·m/m^2)		—	23.8 (19.3–31.0)	24.5 (19.8–34.9)	33.3† (25.0–43.1)	41.1‡ (31.4–50.0)	<0.001

*p <0.05, †p <0.001, ‡p <0.01 for all tests performed versus baseline. Baseline values were at intensive care unit (ICU) admission for temperature, epinephrine infusion, and heart rate; time 0 for mean arterial pressure (MAP); and time 1 for the other values. Mean arterial pressure at admission and time 0 was determined by noninvasive methods, and MAP at times 1, 2, 3, and 4 by invasive monitoring. §The p value in the last column refers to nonparametric analysis of variance (Kruskall-Wallis test). Data are presented as the median value (interquartile range).

CI = cardiac index; HR = heart rate; LVSW = left ventricular stroke work; MPAP = mean pulmonary artery pressure; POAP = pulmonary occlusion arterial pressure; PVRI = pulmonary vascular resistance index; RAP = right arterial pressure; SI = stroke index; SVRI = systemic vascular resistance index.

Among the mechanisms underlying early post-resuscitation arrhythmia and myocardial dysfunction, cytosolic and mitochondrial Ca^{2+} overload following cardiac arrest and CPR has been recognized as a determinant *(Ayoub 2008, Gazmuri 2012)*. Ca^{2+} overload after cardiac arrest is related to myocyte Na^+ content alteration. Under normoxic conditions the late Na^+ current (I_{NaL}) contributes very little to the total Na^+ content of the myocardial cell. However, during ischemia the I_{NaL} channel does not close properly. Under this condition the influx of Na^+ becomes substantial *(Kloner 2011)*. Indeed, Na^+ influx through the late Na^+ channel appears to be the major contributor to the rise of cardiomyocyte intracellular Na^+ concentration observed during ischemia *(Zaza 2008)*. It has been shown, in fact, that ischemia increases the amplitude of I_{NaL} in rat ventricular myocytes, from 50–100 pA up to

180–205 pA. Furthermore, following reperfusion, production of ROS is known to further increase I_{NaL} *(Ma 2005, Slezak 1995, Song 2006)*.

In the setting of cardiac arrest and CPR, main routes for cardiomyocyte Na^+ entry include the sodium-hydrogen exchanger isoform-1, the voltage-gated Na^+ channel, and the Na^+-bicarbonate co-transporter. This cytosolic Na^+ accumulation, further augmented by the concurrent ischemia-induced Na^+-K^+-ATPase inability to extrude Na^+, represents an important pathophysiological mechanism responsible for cell injury *(Wang 2007)*. Cytosolic Na^+ causes, in fact, a subsequent increase in myocyte intracellular Ca^{2+} via the activity of Na^+-Ca^{2+} exchanger (**Figure 8**) *(Ayoub 2008)*.

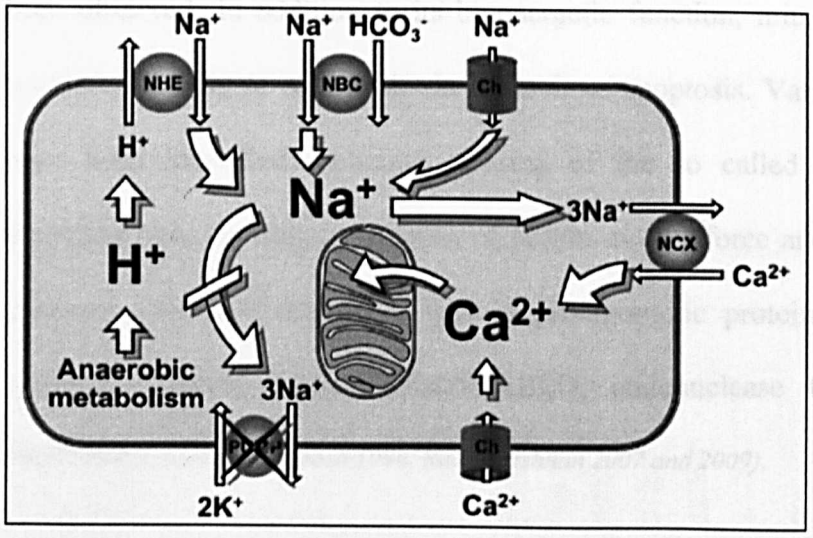


Figure 8. Events leading to cardiomyocyte Ca^{2+} overload during ischemia/reperfusion. NHE = Na^+/ H^+ exchanger; NBC = Na^+/ HBO_3^- exchanger; NCX = Na^+/ Ca^{2+} exchanger; Ch = channel. From *Ayoub 2008*.

Post-resuscitation cytosolic Ca^{2+} accumulation in cardiomyocytes has, indeed, several deleterious consequences, including: electrical instability with ventricular arrhythmias early after resuscitation, i.e. premature ventricular complexes, VT and VF; varying degrees of mechanical LV dysfunction that can compromise hemodynamic function, i.e. reduced contractility and increased diastolic tension; and mitochondrial dysfunction *(Ayoub 2008 and 2010, Gralinski 1996)*.

Mitochondria are the major contributors in many cellular as well as extracellular regulatory functions that affect survival following an ischemic insult. In the setting of cardiac arrest, mitochondria are thought to be progressively damaged during ischemia and further injured when reperfusion resumes. This leads to important alterations of the function of this organelle. Specifically, the dysfunction has been shown to be mainly associated with impaired complex activities that begin to deteriorate within the initial 15 min of ischemia. Nevertheless, ultrastructural changes of mitochondria during ischemia, including swelling, loss of matrix density and cristae disintegration, have been also demonstrated to correlate well with the duration of cardiac arrest *(Yeh 2009, Xu 2010)*. More specifically, within the first hours during the early recovery phase, a greater decrease in respiratory control ratio has been observed. In addition to its bioenergetic function, mitochondria also participate in processes leading to cell death via necrosis or apoptosis. Various distinctive mechanisms have been identified including opening of the so called mitochondrial permeability transition pore (leading to collapse of proton motive force and uncoupling of respiration) *(Halestrap 2004)* and release of various pro-apoptotic proteins, including cytochrome c, apoptosis-inducing factor, Smac/DIABLO, endonuclease G, and a serine protease Omi/HtrA2 *(Cai 1998, Green 1998, Radhakrishnan 2007 and 2009)*.

Post-cardiac arrest systemic inflammatory response

Current guidelines for Cardiopulmonary Resuscitation and Emergency Cardiovascular Care recognize the post-cardiac arrest syndrome has a sepsis-like syndrome *(Adrie 2002, Nolan 2008, Peberdy 2010)*. The whole-body ischemia/reperfusion that follows cardiac arrest with associated oxygen debt causes, in fact, generalized activation of immunological and coagulation pathways, increasing the risk of multiple organ failure and infection *(Adrie 2002 and 2004 and 2005, Adams 2006, Cerchairi 1993b, Esmon 2003)*. As early as 3 hr after cardiac arrest, blood concentrations of various cytokines, soluble receptors, and endotoxin increase, and the magnitude of these changes are associated with outcome. Soluble intercellular adhesion

molecule-1, soluble vascular-cell adhesion molecule-1, and P- and E-selectins are increased during and after CPR, suggesting leucocyte activation or endothelial injury *(Adrie 2002 and 2004, Geppert 2000, Gando 2000)*. Altogether, the high levels of circulating cytokines, the presence of endotoxin in plasma, and the dysregulated production of cytokines found in cardiac arrest patients resemble indeed the immunological profile of patients with sepsis **(Figures 9 and 10)** *(Adrie 2002 and 2004)*.

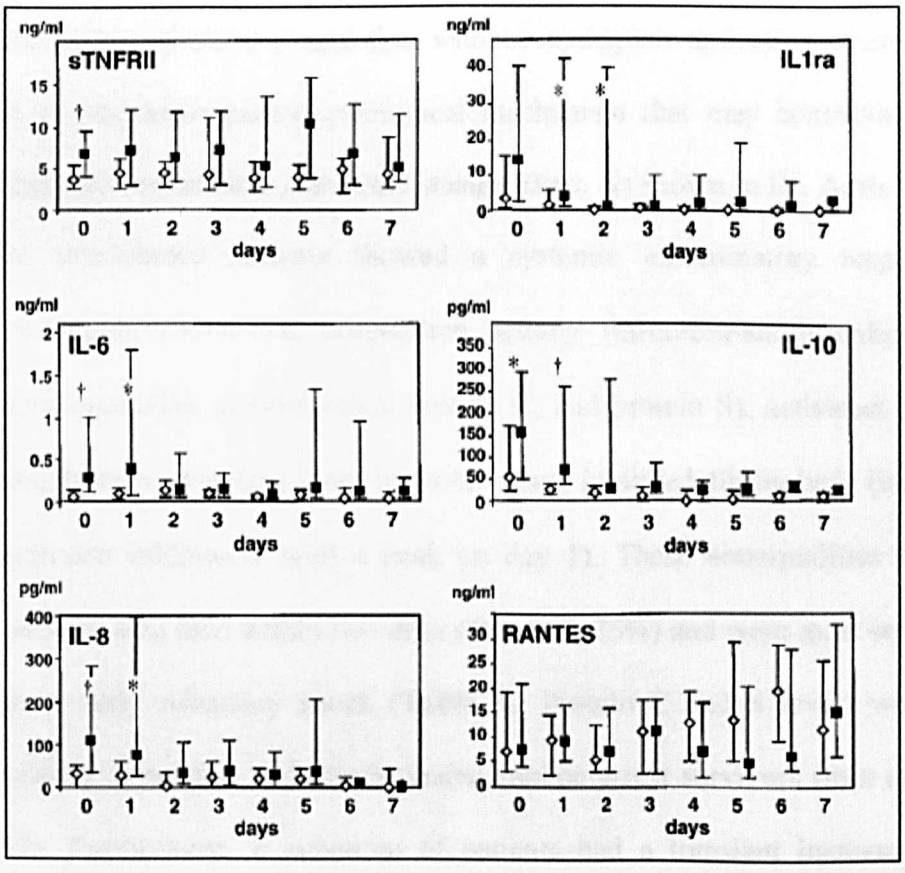


Figure 9. Kinetics of cytokines and sTNFRII levels on admission and over a 7-day period in 61 resuscitated survivors (n=18,open diamonds) and nonsurvivors (n=43, black squares) of cardiac arrest. Data are expressed as median (25% to 75% quartile).
**P*<0.05; †*P*<0.005.
From *Adrie 2002.*

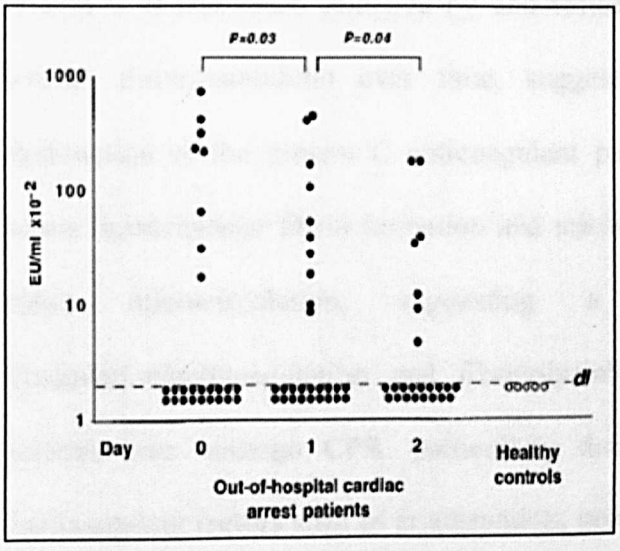


Figure 10. Measurement of endotoxin levels (endotoxin units [EU]/mL) in plasma of 35 resuscitated OHCA patients on admission (day 0) and the 2 days after (days 1 and 2).
dl indicates detection limit (0.02 EU/mL).
From *Adrie 2002.*

The post-cardiac arrest syndrome seems to be causally related to an early systemic inflammatory response, leading to an inflammatory imbalance *(Adrie 2004, Geppert 2000)*, and is also associated with an "endotoxin tolerance," as observed in severe sepsis *(Munoz 1991)*. Additional disturbances, i.e. activation of the coagulation cascade *(Böttiger 1995, Gando 1997)*, platelet activation with formation of thromboxane A2 *(Gando 1997b)*, and an alteration of soluble E-selectin and P-selectin *(Geppert 2000)* have been described.

Activation of blood coagulation without inadequate activation of endogenous fibrinolysis is an important pathophysiological mechanism that may contribute to microcirculatory reperfusion disorders *(Adrie 2005, Böttiger 1995)*. As shown in Dr. Adrie's work, at admission, 67 resuscitated patients showed a systemic inflammatory response with increased interleukin (IL)-6 and coagulation activity (thrombin-antithrombin complex), reduced anticoagulation (antithrombin, protein C, and protein S), activated fibrinolysis (plasmin-antiplasmin complex), and, in some cases, inhibited fibrinolysis (increased plasminogen activator inhibitor-1 with a peak on day 1). These abnormalities were more severe in patients who died within two days (50 of 67, 75%) and were most severe in patients dying from early refractory shock (**Table 3**). Protein C and S levels were low compared to healthy volunteers and discriminated cardiac arrest survivors from non-survivors (**Figure 11**). Furthermore, a subgroup of patients had a transient increase in plasma-activated protein C at admission followed by undetectable levels. This, along with an increase in soluble thrombomodulin over time, suggested a secondary endothelial injury and dysfunction of the protein C anticoagulant pathway similar to that observed in severe sepsis. Intravascular fibrin formation and microthromboses are distributed throughout the entire microcirculation, suggesting a potential role for interventions. Coagulation/anticoagulation and fibrinolysis/antifibrinolysis systems are activated in patients who undergo CPR, particularly those who recover spontaneous circulation. Anticoagulant factors such as antithrombin, protein S, and protein C are decreased and are

associated with a very transient increase in endogenous activated protein C soon after the cardiac arrest/resuscitation event. Finally, the stress of total body ischemia/reperfusion affects adrenal function. Although an increased plasma cortisol level occurs in many patients after out-of-hospital cardiac arrest, relative adrenal insufficiency, defined as failure to respond to corticotrophin, is common *(Hekimian 2004, Schultz 1993)*.

Table 3. Coagulation Parameters in Healthy Volunteers (Negative Control Group), Patients With Septic Shock (Positive Control Group), and Patients Admitted After Successfully Resuscitated Out-Of-Hospital Cardiac Arrest

Parameters	Healthy Volunteers (n = 10)	Patients With Severe Sepsis (n = 12)	Cardiac Arrest Patients			p Value*
			At ICU Admission (n = 67)	Day 1 (n = 59)	Day 2 (n = 50)	
White blood cell counts ($\times 10^9$/l)	7.2 (6.6–7.8)	12 (7.3–30)	14.5 (11.3–18.7)	15.1 (10.3–21)	13.1 (9.6–17.1)	10^{-4}
Hematocrit (%)	42.3 (42–44)	32 (30–35)	42 (39–46)	42 (37–45)	36 (35–40)	10^{-4}
Platelets ($\times 10^9$/l)	255 (223–304)	168 (92–213)	215 (176–280)	192 (155–262)	151 (117–190)	10^{-4}
IL-6 (pg/ml)	0 (0–0)	713 (259–884)	158 (53–400)	216 (72–811)	106 (38–415)	0.51
Protein C (%)	125 (100–140)	35 (27–44)	70 (34–100)	73 (25–99)	76 (32–102)	0.16
Protein S (%)	103 (92–115)	41 (34–56)	60 (40–78)	53 (22–71)	62 (33–82)	0.32
sTM (ng/ml)	45 (40–53)	100 (70–123)	40 (32–56)	53 (36–89)	59 (40–119)	10^{-4}
AT (%)	107 (107–110)	51 (41–139)	88 (74–99)	88 (78–95)	82 (70–101)	0.003
D-dimer (μg/ml)	0.25 (0.22–0.32)	4 (3.5–7)	9 (2.6–20)	3.7 (2–15)	2.2 (1.1–4)	10^{-4}
PT (s)	12.9 (12.4–13.1)	21 (19–28)	19.6 (15.2–28)	18.6 (15.5–30.7)	20.3 (15.5–28.3)	0.66
APTT (s)	39 (36–41)	67 (55–71)	43.8 (36–54)	54.6 (41–70)	59.8 (45–74)	0.01
TAT complex (μg/ml)	1.8 (1.8–2.1)	9.7 (6–15)	36.2 (14.8–122)	11.9 (6.7–32.6)	7.6 (4.7–13.3)	10^{-4}
PAP complex (μg/ml)	441 (289–554)	528 (450–740)	2,754 (1,654–4,576)	899 (450–1,749)	607 (415–996)	10^{-4}
PAP/TAT ratio	235 (169–282)	53 (36–122)	76 (39–133)	71 (41–124)	102 (43–132)	0.32
PAI-1 (AU/ml)	7.5 (5.7–10)	37 (24–86)	22.5 (7.3–43)	40.1 (22–86)	25 (15–40)	0.04

*Data are expressed as median (interquartile ranges). p values are for comparisons between healthy volunteers, septic patients, and cardiac arrest patients at admission. Normal ranges and abbreviations of each biomarker are reported in the Appendix.

Interleukin-6 (IL-6), Soluble thrombomodulin (sTM), Antithrombin (AT), Prothrombin time (PT), Activated partial thromboplastin time (APTT), Thrombin-antithrombin complex (TAT), Plasmin-antiplasmin complex (PAP), Plasminogen activator inhibitor-1 (PAI-1). *From Adrie 2005.*

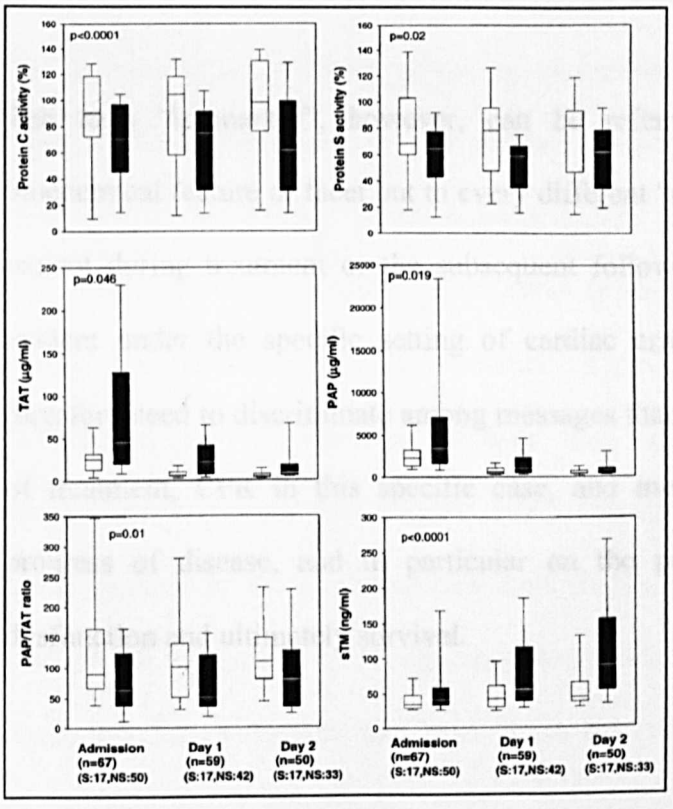


Figure 11. Changes in coagulation over the first two intensive care unit days in 67 patients successfully resuscitated after out-of-hospital cardiac arrest, 17 survivors (S) **(open boxes)** and 50 nonsurvivors (NS) **(black boxes)**. Patients receiving oral anticoagulation were excluded from the protein C and S graphs.
Medians are shown as **lines**, 25th to 75th percentiles as **boxes**, and 5th to 95th ranges as **error bars**.
PAP=plasmin-antiplasmin complex; sTM=soluble thrombomodulin; TAT=thrombin-antithrombin complex.
From Adrie 2005

BIOMARKERS TO PREDICT OUTCOME OF CARDIAC ARREST

Biomarkers are measurable and quantifiable biological parameters which serve as indices for health and disease in the individual patient. The prognosis of patients resuscitated from cardiac arrest can be estimated by information obtained from the clinical history, ECG abnormalities, and, more recently, from biochemical indicators of myocardial injury and dysfunction, as well as markers of renal failure and inflammatory activity *(James 2003)*. In the past 2 decades, biomarkers have become an increasingly important tool in clinical practice, helping to improve patient care. For example, biomarkers have demonstrated a significant impact in early detection of sub-clinical disease (i.e., prostate-specific antigen screening for prostate cancer), diagnosis of acute or chronic syndromes (i.e., B-type natriuretic peptide in heart failure, cardiac troponins for myocardial infarction), risk stratification (i.e., cardiac troponin in acute coronary syndromes), and monitoring of disease or therapy (i.e., hemoglobin A1c in diabetes mellitus). Apart from oncology, cardiovascular medicine is the arena in which novel biomarkers have been most extensively evaluated. However, only a minority of markers has demonstrated significant diagnostic and/or therapeutic impact *(Hochholzer 2010)*.

The term "biomarker", however, can be referred not only to a dosable humoral biochemical feature or facet but to every different "message" that can be assessed from the patient during treatment or the subsequent follow-up. Indeed, this anticipation is more evident under the specific setting of cardiac arrest. For this particular condition, we therefore need to discriminate among messages that provide feedback regarding the effects of treatment, CPR in this specific case, and messages that bring information on the progress of disease, and in particular on the post-resuscitation cardiac and cerebral dysfunction and ultimately survival.

Circulating biomarkers

Circulating biomarkers have become an increasingly important tool in clinical practice, useful for diagnosis, risk stratification, monitoring, and prognosis of patients. Predicting survival and especially the neurological outcome after ROSC in victims of cardiac arrest still remains a difficult issue. The decision to continue, limit or stop intensive care is a major problem for deep ethical implications and also for resources allocation. Accurate prediction of the neurologic outcome of comatose patients following cardiac arrest is therefore essential to establish the model of care *(Thenayan 2008)*. Prognostication of outcome in cardiac arrest is of importance because it could help physicians to make decisions and enhance therapeutic efforts in patients with predictable overall good recovery and to readdress the overall management or ultimately to make a decision on withdrawal of care in those with expected poor outcome *(Bouwes 2012)*. A procedure used to support no-treatment decisions, however, should ideally have no false-positive results *(Wijdicks 2006, Zandbergen 2001)* reaching a specificity of 100% for poor outcome. A false prediction of a poor outcome may cause the patient to be denied life supporting treatment. On the other hand, a falsely optimistic prediction, although less serious from an ethical point of view, may lead to unnecessary prolongation of costly therapy. Due to the heterogeneity of cardiac arrest population and scenarios, no single factor has been identified as a reliable predictor of outcome. In addition, with recent advances in the use of therapeutic hypothermia (TH), prediction of patients' outcome has become even more challenging. In fact, there is evidence that TH makes the well-established tests in normothermic patients less reliable. Early assessment of patients brain damage remains quite difficult in the intensive care unit (ICU), and could be actually reachable only through the integration of the answer given by clinical examination, neuro-functional and neuro-imaging evaluation, and biochemical markers dosing *(Sandroni 2013 and 2013b)*. This

evaluation should be carried out after the initial 72 hr post-ROSC. Normothermic and hypothermic patients with a favorable prognosis predominantly regain consciousness within 3 days *(Fugate 2011)*. A reliable prediction in TH is, however, quite difficult. Likewise, a residual effect of sedative and analgesic substances must be excluded, due to longer drugs' half-life during TH. Indeed, a significantly delayed awakening in patients treated with TH could be observed *(Lurie 2011)*. For this reason, the early termination of life support 72 hr after re-warming needs to be reconsidered critically and is not indicated.

The current clinically used biomarkers to predict outcome of cardiac arrest are cTns and creatine kinases (CKs) for the heart, and NSE and S-100b, for the brain *(Peberdy 2010, Scolletta 2012)*. Nevertheless, evidence does not support the use of these serum biomarkers alone as predictors of outcome in the setting of cardiac arrest *(Neumar 2010, Nolan 2010)*. Furthermore, the use of the above markers is an adoption from conditions of "localized" myocardial or brain ischemic insults, while cardiac arrest is a wider and more complex event, involving the whole body. Accordingly, research in this domain extends from localized to generalized ischemic injury, a condition of "global" ischemia and reperfusion *(Becker 2002)*.

A novel investigating approach, that considers mechanisms of both injury and preservation and that is able to provide feedback regarding the effects of treatment, and the progress of the disease, particularly post-resuscitation cardiac and neurological dysfunction, is advocated. Up to date cardiac, neurological and inflammatory biomarkers, as potential predictors of outcome after cardiac arrest, are summarized in **Table 4** *(Scolletta 2012)*.

Table 4. Most commonly used biomarkers of organ injury after cardiac arrest.

Biomarker	Use	Cutoff	Timing	Sensitivity (%)	Specificity (%)	FPR	TH	Limitations
Heart injury								
Troponin	AMI Diagnosis	0.6–14.5 ng/ml	< 12 h	72–88	75–95	NA	No effects	Need to be combined with ECG Specific only if ST-elevation Elevated after coronary angiography
CK-MB	AMI Diagnosis	60 ng/ml	< 12 h	88	88	NA	NA	Not specific of AMI Elevated because of CPR Elevated because of defibrillation
BNP	Outcome	80–230 pg/ml	Admission	83–87	87–96	NA	NA	Limited studies Elevation in other conditions with HF
Brain injury								
NSE	Outcome	> 33 mg/l	< 72 h	72–80	84–100	0–23%	Reduced levels	Elevated if hemolysis Elevated if use of LVAD/IABP Elevated in some cancers
S100B	Outcome	0.2–1.5 mg/l	< 72 h	70–80	85–100	0–16%	NA	Elevated if heart and aorta injury Elevated in some cancers
Inflammation								
Procalcitonin	Outcome	0.5–1.0 ng/ml	< 24 h	70–85	80	NA	NA	Elevated in inflammatory conditions Elevated if underlying infection

AMI: Acute myocardial infarction; BNP: Brain natriuretic peptide; CK-MB: Creatine kinase-MB; CPR: Cardiopulmonary resuscitation; FPR: False-positive rate; HF: Heart failure; IABP: Intra-aortic balloon pump; LVAD: Left ventricular assist device; NA: Not available; NSE: Neuron-specific enolase; TH: Therapeutic hypothermia.

From Scolletta 2012.

CIRCULATING BIOMARKERS OF CARDIAC INJURY

Within the last decade a broad range of circulating markers associated with an increased risk for death and cardiovascular endpoints have been identified (**Table 5**). Adding to markers of cell necrosis are markers of ischemia, inflammation, plaque destabilization or rupture, myocardial dysfunction, and stress. As shown in Table 5, most of these markers have demonstrated at least some prognostic value *(Hochholzer 2010)*.

Comparison of selected cardiovascular biomarkers

Table 5

	Prognostic impact	Diagnostic impact	Therapeutic impact
Markers of necrosis			
Creatine phosphokinase MB	+++	+++	++
Myoglobin	++	++	++
Troponin	++++	++++	++++
Markers of myocardial dysfunction or stress			
Atrial natriuretic peptides	+++	+++*	?
Brain natriuretic peptides	++++	++++*	+++*
Copeptin	++	+	?
Proadrenomedullin	++	+	?
Markers of inflammation			
Adiponectin	++	?	?
C-reactive protein	++++	?	++
Growth differentiation factor 15	+++	?	+
Interleukin 6	+++	?	?
Soluble ST2	+	?	?
Tumor necrosis factor α	++	?	?
Myeloid-related protein 8/14	+	?	?
Markers of ischemia			
Choline	++	?	?
Heart-type fatty acid-binding protein	++	++	?
Ischemia modified albumin	+	+	?
Markers of plaque destabilization/rupture			
Lipoprotein-associated phospholipase A2	+++	?	?
Matrix metalloproteinase-9	++	?	?
Myeloperoxidase	+++	++	?
Placental growth factor	++	?	?
Pregnancy-associated plasma protein A	+++	+	?
Secretory phospholipase A2	+	?	?
Soluble fms-like tyrosine kinase 1	+	+	?
Soluble intercellular adhesion molecule 1	+++	?	?
Markers of platelet activation			
Soluble CD40 ligand	++/?	?	?
Soluble P-selectin	++	?	?

+, Some evidence by small studies; ++, intermediate evidence from several studies or one large study or trial; +++, good evidence from several large studies or trials; ++++, excellent evidence; ?, conflicting results or no results available or not applicable.
This table only gives an overview of the evidence published for the various markers. It does not indicate the clinical utility of different markers (eg, a marker might be very useful for risk stratification, but not feasible for the clinical setting due to limitations in detection or because it is also elevated at important differential diagnoses).
* For stratification of patients with heart failure.

From *Hochholzer 2010.*

For patients resuscitated from cardiac arrest, it is important to determine a definite cause, that is, myocardial ischemia vs. primary ventricular arrhythmia, owing to the risk of recurrence and the different therapeutic approaches available. Acute myocardial infarction (AMI) may account for up to 50% of out-of-hospital cardiac arrest, ischemic heart disease without AMI for one-third of cases and non-ischemic cardiomyopathy for nearly 10% of all arrests *(de Vreede-Swagemakers 1998, Voicu 2012)*. cTns, CKs and natriuretic peptides are the most used biomarkers of myocardial injury/dysfunction.

Troponins

Troponin I, C, and T form a complex that regulates the Ca^{2+}-modulated interaction of actin and myosin in striated muscle. Among the cardiac markers, cTns I and T are sensitive and

specific markers of myocardial injury and are used routinely for the diagnosis of acute coronary syndromes. They provide prognostic information and are of great value for risk stratification of patients *(Antman 1996, Apple 2005, Aviles 2002, Donnelly 1998, Lindahl 2000).* Elevated cTn blood levels have been reported in several cohorts of patients with heart failure, and the magnitude of elevation has been correlated with the severity of the disease and with adverse outcomes *(Ishii 2002, Setsuta 1999, Sato 2001).* Because of their high cardiac specificity, elevated blood cTns may suggest ongoing myocardial damage and may serve as a marker for the progression of disease during the post reperfusion recovery. Currently, for every patient who has suffered a myocardial ischemic event or who has been resuscitated from cardiac arrest, cTn T or I are considered the first-line test *(Williams 2005).* Cardiac troponin T has been investigated extensively and has been found to be a sensitive marker of myocardial necrosis *(Katus 1991 and 1992, Mair 1992).* The presence of elevated levels of cTnT in the general population has a prevalence of less than 1% and this condition is commonly associated with an underlying cardiovascular disease or high-risk phenotypes for cardiac accidents, especially in persons with chronic heart failure. Currently, new highly sensitive assays for determination of cTns are available and have shown that cTnT retains a prognostic value at previously undetectable concentrations*(Latini 2007).* When cTnT levels were investigated in more than 4.000 patients with a LV ejection fraction (EF) of <40% using both the standard assay and the high sensitivity assay, cTnT detection increased from approximately 10% of the population to more than 90%. The circulating concentration of this highly sensitive (hs) cTnT showed even greater prognostic accuracy in association with increases of another biomarker, namely brain natriuretic peptide (BNP). In 658 patients who presented with BNP above the normal median concentration and cTnT below, mortality was 14%. However, in an additional 632 patients with BNP below the median and cTnT above, mortality was 20%. Finally, in the 1.331 patients with both markers above their respective median concentrations, mortality increased to 32% *(Latini 2007).* A continuous, slow release of cTns from the myocardium

might reflect an ongoing cardiac myocyte cell death. This condition has been associated with the condition of LV dysfunction following myocardial ischemia in both animal models and humans patients suffering with chronic heart failure *(Narula 1999, Olivetti 1997)*. If ongoing cardiac damage at a very low rate is the determinant of these circulating troponins, other mechanisms may, however, account for this phenomenon, such as stretching of cardiac myocytes with transient loss of cell membrane integrity. There are few investigations on the role of cTns for the differential diagnosis of AMI in patients successfully resuscitated after out-of-hospital cardiac arrest. Early studies reported a substantial lack of sensitivity and specificity *(Müllner 1996)*. This might have been due to the fact that procedures used for CPR (CC or DF) or persistent circulatory shock might have directly provoked myocardial damage and the release of cardiac markers, in the absence of a coronary artery occlusion *(Müllner 1998)*. Troponin elevation in patients resuscitated from cardiac arrest but who do not have AMI is however lower and normalizes faster than after AMI *(Oh 2012)*. In a recent, single-centre study, 163 patients resuscitated from cardiac arrest were assessed with coronary angiography on admission for AMI, that was diagnosed in 37% of the cases *(Voicu 2012)*. High circulating cTn concentrations were measured very early after cardiac arrest, even in patients with normal angiograms (median cTnI was 0.6 ng/mL in the latter group), indicative of non-ischemic myocardial injury during CC. However, combined with ST-elevation on ECG, elevated cTn concentration on admission (cTnI> 2.5 ng/mL) showed a good performance to exclude the diagnosis of AMI (sensitivity 93%, negative predictive value 94%). The specificity remained low even in combination with ST-elevation on ECG (64%).

International guidelines recommend considering emergent coronary angiography and percutaneous coronary intervention in cardiac arrest patients after ROSC, even in the absence of ST elevated myocardial infarcton (STEMI) *(Peberdy 2010)*. Since clinical findings such as chest pain are often lacking and the predictive value of ECG for AMI is

poor, cTn testing may provide a simple and objective selection of post-resuscitation patient candidate for immediate coronary angiography. This triage strategy has recently been tested in 422 cardiac arrest survivors without obvious extra-cardiac causes *(Dumas 2012)*. In this large study, a coronary angiography was systematically performed and cTn measured on admission. However, even if independently associated with coronary occlusion, elevation of cTn levels had a poor accuracy to identify a recent coronary lesion, precluding its use as the sole criteria for the decision to perform or not early coronary angiography in these patients (**Figure 12**).

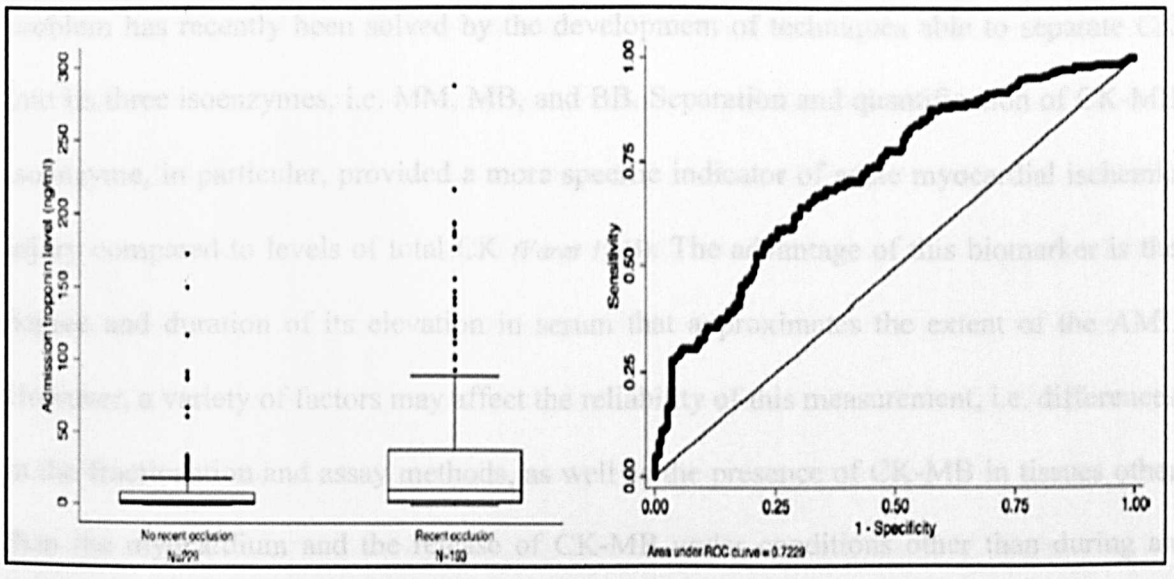


Figure 12. Troponin levels dispatched according to the presence of a recent coronary lesion. Troponin I receiving operating characteristic (*ROC*) curve predicting a recent coronary lesion. From *Dumas 2012*.

Acute coronary artery occlusion is therefore difficult to diagnose in survivors of cardiac arrest using circulating markers alone, especially in case of ambiguous ECG. There are no data available to date on the diagnostic performance of cTns after cardiac arrest, when using high sensitivity assays that may readily detect concentrations in the order of few nanograms per liter, ten to hundred times lower than the conventional assays. However, one may speculate that specificity will become an even more critical issue since the recent introduction of this new generation of high sensitivity reagents has led to a substantial

increase in the proportion of detectable cTn levels attributable to conditions distinct from acute coronary syndromes *(de Lemos 2013)*. Moreover, the diagnostic value of cTns could be altered by CPR. Increased c-Tn levels were, in fact, reported in approximately 40% of cardiac arrest patients, when samples were drawn during CPR *(Lai 2004)*.

Creatine kinase

Measurement of CKs in the serum was extensively used in the 1970s as a tool in the diagnosis of AMI. In a large number of patients, however, an elevated CK value added little information because of the presence of concomitant skeletal muscle damage. This problem has recently been solved by the development of techniques able to separate CK into its three isoenzymes, i.e. MM, MB, and BB. Separation and quantification of CK-MB isoenzyme, in particular, provided a more specific indicator of acute myocardial ischemic injury compared to levels of total CK *(Varat 1975)*. The advantage of this biomarker is the degree and duration of its elevation in serum that approximates the extent of the AMI. However, a variety of factors may affect the reliability of this measurement, i.e. differences in the fractionation and assay methods, as well as the presence of CK-MB in tissues other than the myocardium and the release of CK-MB under conditions other than during an AMI *(Guzy 1977)*. At present, measurement of CK-MB is an alternative to cTns measurement, but is only recommended, if cTn measurement is not available *(Jaffe 2000)*. CK-MB levels have been reported to be significantly higher in cardiac arrest patients having a AMI than in those without AMI. Indeed, a cutoff of 60 ng/ml for CK-MB had a sensitivity/specificity of 88% to diagnose AMI after cardiac arrest *(Grubb 1996, Sideris 2011)*. Nevertheless, release of CK-MB may also occur because of CPR, in the absence of any significant coronary lesion *(Oh 2012)*. As for cTns, more than 75% of cardiac arrest survivors may present an elevation in CK-MB levels within 24 hr, which is not only associated with ischemic myocardial damage, but also with cumulative energy delivered during DF and with the duration of CPR *(Mattana 1992, Müllner 1996)*.

Brain natriuretic peptide

The stretching but also ischemia of cardiomyocytes occurring during cardiac arrest induces the release of natriuretic peptides, especially the brain natriuretic peptide (BNP), which are commonly used in the diagnosis, therapy monitoring and risk stratification of several clinical conditions, such as heart failure, acute coronary syndromes, weaning from mechanical ventilation *(Prahash 2004)*. B-type natriuretic peptide and its more stable counterpart, N-terminal pro-B-type natriuretic peptide, have shown great promise, covering a wide range of acute coronary syndromes *(Omland 2002)*. BNP has numerous advantages compared to other biomarkers. BNP levels allow for accurate diagnosis of heart failure and may be helpful to screen for asymptomatic LV dysfunction in high-risk patients. BNP levels are also important tools for risk stratification of patients, prediction of death, and in combination with symptoms and signs, assessment of clinical decompensation *(Maisel 2008)*. Natriuretic peptides have been tested for their relation to survival or neurological outcome after cardiac arrest. Nagao et al. measured BNP on arrival at emergency room in 401 cardiac arrest patients with presumed cardiac origin *(Nagao 2004)*. Primary outcome was survival to hospital discharge, and among the secondary outcomes was neurological evaluation according to the CPC scale. Mean BNP was significantly higher in the decedents (260 pg/mL) than in the 52 survivors (74 pg/mL). Survival decreased steeply across the quartiles of BNP concentration, being 34, 10, 7 and 1%, respectively from bottom to top. The same trends were observed in patients with witnessed arrest, CPR by bystander, with shockable rhythm or with ROSC after arrival at emergency room. Higher BNP levels were significantly associated to death, even after adjustment for variables associated with survival (witnessed arrest and ROSC before hospital), suggesting a role for BNP for risk stratification of survival of resuscitated patients. The highest prognostic accuracy was observed at a BNP concentration of 100 pg/mL, that corresponded to a negative predictive value (NPV) of 96% and a specificity of 66%. Finally, the proportion of patients with favorable neurological outcome (good recovery or moderate disability)

decreased from 33% in the bottom quartile of BNP concentration to 0% in the top one *(Nagao 2004)*. More recently, the same group evaluated BNP to predict neurological outcome in comatose survivors of cardiac arrest due to cardiac causes and treated with mild TH by extracorporeal cooling *(Nagao 2007)*. The primary endpoint was a favorable neurological outcome at the time of hospital discharge. There was a rapid fall in the proportion of patients with favorable neurological outcome across the quartiles of BNP concentration, in all patients, and in those with witnessed arrest, bystander CPR, shockable rhythm, ROSC after arrival at emergency room or cardiac arrest due to acute coronary syndrome. The fact that BNP levels measured on admission in comatose cardiac arrest survivors may predict neurological outcome has been confirmed in an independent study that enrolled 115 patients followed for 6 months *(Sodeck 2007)*. In this setting, BNP was significantly associated with an adverse neurological outcome and mortality, independent of the pre-arrest health and cardiac conditions. Though BNP provides invaluable information on the post-resuscitation cardiovascular function, and therefore indirectly on cerebral perfusion, it is however clear that brain-specific markers, such as NSE or S-100b, are more promising candidates for the prediction of neurological outcome after successful cardiopulmonary resuscitation *(Shinozaki 2009)*.

CIRCULATING BIOMARKERS OF BRAIN INJURY

Biochemical markers of brain injury are logical choices as prognostic markers of neurological outcome. Of these biomarkers NSE and S-100b are among the most widely studied and used. Considering the different characteristics of these two biomarkers, a possible rationale for analyzing both molecules in patients after cardiac arrest is their different distribution within the grey (NSE) and white (S-100b) matter.

Neuron-specific enolase

NSE is a neuron-derived enzyme, which is released after stroke and cardiac arrest and can be detected in the blood. Its level in serum correlates with the extent of the neurological damage. NSE is a 78-kDa with a half-life of 24 hr, intracellular enzyme found in neurons and other cells of neuroectodermal origin. Elevation of serum NSE 1–3 days after cardiac arrest is regarded as a severity marker of post-anoxic neuronal injury. The American Academy of Neurology, which considered studies from the pre-hypothermia era, suggested a cutoff of > 33 ug/L at days 1–3 after ROSC as a robust predictor for poor outcome *(Wijdicks 2006)*; the reported false positive rate (FPR) varied from 0 to 3%. Cerebral hypoxia causes death of neuronal cells as well as damage to the BBB, resulting in an elevation of the serum levels of this and other markers. The serum levels often correlated with the extent of brain damage and in some studies with the prognosis of the patients. The predictive value of NSE in patients with ROSC has been shown in several trials prior to the use of TH *(Reisinger 2007, Zandbergen 2006)*. An elevation of NSE was associated with poor outcome for comatose patients after cardiac arrest *(Auer 2006, Grubb 2007, Meynaar 2003, Roine 1989)*. Fogel et al. *(Fogel 1997)* reported that NSE concentrations exceeding 33 µg/l at any time within a week since arrest had a sensitivity of 80% and specificity of 100% to predict persistent coma. Also, Zandbergen et al. *(Zandbergen 2006)* found in a large cohort of resuscitated patients that NSE concentrations above 33 µg/l within 72 hr after arrest predicted poor outcome with a FPR of 0%. However, a subgroup analysis of the Hypothermia after Cardiac Arrest trial *(Tiainen 2003)* showed a decreased prognostic value of NSE in patients treated with TH. Since the introduction of the 2010 guidelines, various studies have dealt with NSE during TH. Although an elevated NSE predicts a poor outcome, there are case reports of good neurological survival despite extremely high NSE levels in serum *(Grubb 2007, Krumnikl 2002)*. In a prospective observational study on 97 patients, patients with poor outcome at 3 months had significantly higher NSE levels at 24 and at 72 hr than those with good neurological. A peak NSE level of 47 µg/l had the

highest specificity (84%) and sensitivity (72%) to predict poor outcome, with a positive predictive value (PPV) of 93%. A cutoff value for NSE of 97 µg/l predicted a poor neurological outcome with a specificity of 100% and a sensitivity of 49% *(Daubin 2011)*, NSE levels >33 µg/l resulted in a rate of false prediction of poor outcome ranging from 7% for measurements taken 48 hr after arrest to 10% if NSE was assessed during TH *(Bouwes 2012)*. Other cutoffs have been also reported, widely ranging from 9 to 91 µg/l *(Reisinger 2007, Zingler 2003)*. It is therefore difficult to identify a specific cut off predictive of neurological outcome.

In addition, there is also evidence for a constant turnover of NSE in blood, making changes specifically associated with brain damage in serum levels difficult to evaluate. For example, hemolysis caused by invasive procedures might produce a false rise in NSE *(Zandbergen 2001)*. Moreover, it is well known that platelets are markedly activated and hemolysis may occur during early reperfusion after cardiac arrest. Therefore, even though at least a proportion of NSE is released from neurons, the determination of NSE, particularly during early reperfusion after cardiac arrest, may not be absolutely brain-specific. More recently, increases of NSE levels over time, i.e. 2 to 6.4 ug/L during the initial 24 and 48 hr after ROSC, have shown better predictivity for poor outcome *(Oksanen 2009, Rundgren 2009)*.

Protein S-100b

S-100b is an astroglial protein, which is also released after stroke and cardiac arrest. S-100b is an acidic protein with a molecular weight of 21 kDa with a Ca^{2+} binding motif, with a half-life of approximately 2 hr. It is expressed in brain astrocytes and in peripheral adipocytes, but can also be detected in serum. A cut-off value more than 0.21 ng/ml at day 1 after cardiac arrest was 100% predictive of death and poor neurological outcome. The time course of S-100b is therefore different from that of NSE (**Figure 13**).

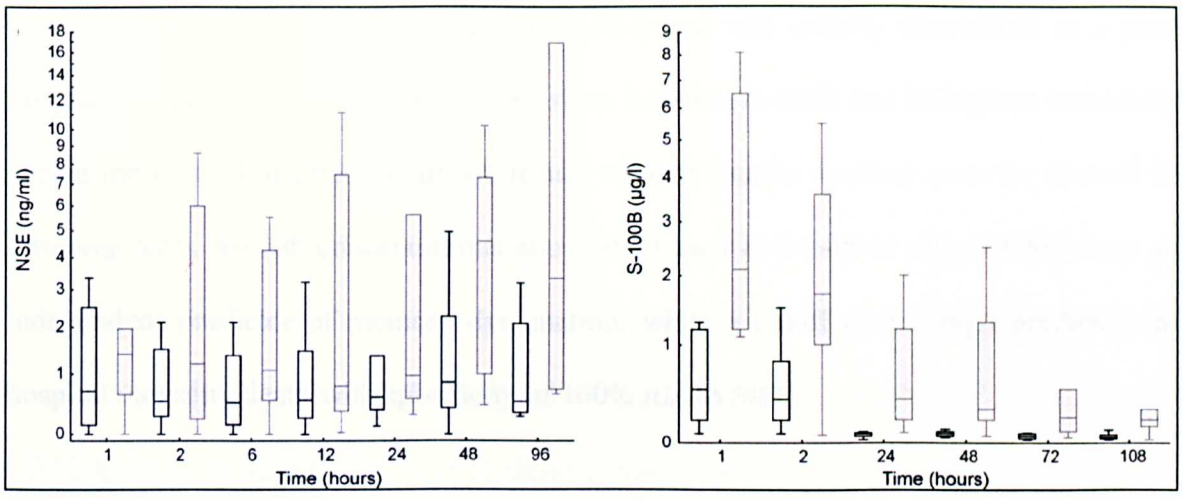


Figure 13. Different time courses and levels of neuron specific enolase (NSE) and S-100b at different time points plotted on logarithmic scales. Line =median; box = interquartile range. Black: Good outcome. Grey, filled: Poor. From *Mörtberg 2011.*

Studies have shown that S-100b can be used to identify patients at risk of significant cognitive impairment *(Rundgren 2009)*. The S-100b protein is implicated in neuronal differentiation and proliferation; there are 19 types of S-100b, among which at least four subtypes are expressed in human tissues: S-100A1 (striated muscles, heart and kidneys), S-100A1B (astroglial cells), S-100B (astroglial and Schwann cells) and S-100BB (astroglial cells). The lower molecular weight, compared with NSE, allows a more rapid and easier release of S-100b across the BBB in the systemic circulation, thus potentially giving a higher sensitivity to detect brain injury. However, the use of S-100b for treatment limitation is controversial. Similar to the NSE, the definition of a cut off is impossible.

Several clinical studies have reported that serum S-100b concentrations at 24 hr after arrest were significantly higher in patients remaining comatose after cardiac arrest than those regaining consciousness. Moreover, increased serum concentration of S-100b predicted extensive post-anoxic brain damage; however, different cutoffs have been proposed, varying from 0.2 to 1.5 mg/L *(Martens 1998, Pfeifer 2005, Rosén 1998)*, depending also on the different time of sampling and on the dosage methods. However, S-100b within the first 2 days after cardiac arrest showed a median FPR of 2–5% in identifying patients with poor

outcome *(Pfeifer 2005, Rosén 1998, Zandbergen 2001)* and was initially considered as a poor prognostic indicator. Because of its very short half-life, S-100b has its highest sensitivity during the first 24 hr after the anoxic insult, while it usually declines over the next 48 hr *(Mörtberg 2011)*. S-100b concentrations above 0.29 mg/l at 24–48 hr after ROSC were an independent predictor of memory dysfunction, while a cutoff of 1.2 mg/l predicted in-hospital mortality death with specificity of 100% *(Grubb 2007)*.

Several studies have compared S-100b and NSE concentrations in the prediction of outcome after cardiac arrest. Some studies reported that serum S-100b was more specific than NSE to predict mortality and poor outcome *(Martens 1998, Mörtberg 2011, Shinozaki 2009b)*. Nevertheless, other studies have yielded conflicting results, reporting similar or a better predictive value for NSE when compared with S-100b *(Rundgren 2009)*. These discrepancies might be related to main differences in the cohort of patients included: origin of cardiac arrest; initial rhythms; witnessed; age.

CIRCULATING BIOMARKERS OF SYSTEMIC INFLAMMATORY RESPONSE

Successful resuscitation from cardiac arrest is usually characterized by the development of an ischemia/reperfusion syndrome of the whole body. The main determinant of this syndrome is a generalized activation of inflammatory reactions resulting in symptoms similar in many aspects to those of sepsis. The main limitation in using inflammatory biomarkers to predict outcome after cardiac arrest is their poor specificity for the anoxic insult, as all inflammatory conditions can increase the circulating levels of such molecules *(Gaussorgues 1988, Nielsen 2011)*.

Procalcitonin

Evidence suggests that procalcitonin (PCT) is not a specific marker of cardiac arrest but of the ischemia/reperfusion process following global ischemia. PCT levels were reported to

be significantly higher in resuscitated patients dying with refractory shock than in those who died with isolated irreversible hypoxic encephalopathy. Some studies have investigated the prognostic role of PCT in comatose survivors. Indeed, PCT was significantly higher in patients with a bad neurological outcome than in those with a good neurological outcome *(Fries 2003, Hayashida 2010)*. More recently, PCT predicted poor outcome with a sensitivity of 85% and a specificity of 81%, for a cutoff of 1 ng/ml. Above a PCT level of 16 ng/ml, no patient regained consciousness *(Stammet 2011)*. In a recent study, early elevations of serum PCT levels correlated with the severity of post-cardiac arrest syndrome and were associated with worse neurological recovery after TH. Unexpectedly, elevated serum PCT did not correlate with early-onset infections in that setting *(Engel 2013)*. Peak PCT correlated with Sequential Organ Failure Assessment (SOFA) score at day 1 and was associated with neurological recovery at 3 months (peak PCT 1.08 [0.35–4.45] ng/ml in patients with CPC 1–2 vs. 3.07 [0.89–9.99] ng/ml in those with CPC 3–5, p = 0.01). However, peak PCT did not differ significantly between patients with early-onset vs. no infections (2.14 [0.49–6.74] vs. 1.53 [0.46–5.38] ng/ml, p = 0.49) *(Annborn 2013)*. In another recent study, serial serum concentrations of PCT were assessed in 84 patients treated with TH after cardiac arrest, and to study their association to severe infections, post-cardiac arrest syndrome and long-term outcome. PCT displayed an early release pattern with a significant increase within 2 hr, increasing further at 6 hr in patients with poor outcome. PCT was strongly associated with SOFA score and time to ROSC, and predicted poor neurologic outcome with high accuracy (area under the receiver operating characteristic (ROC) curve of 0.88, 0.86 and 0.87 at 12, 24 and 48 hr respectively) (**Figure 14**). Again, no association of PCT to infection was observed.

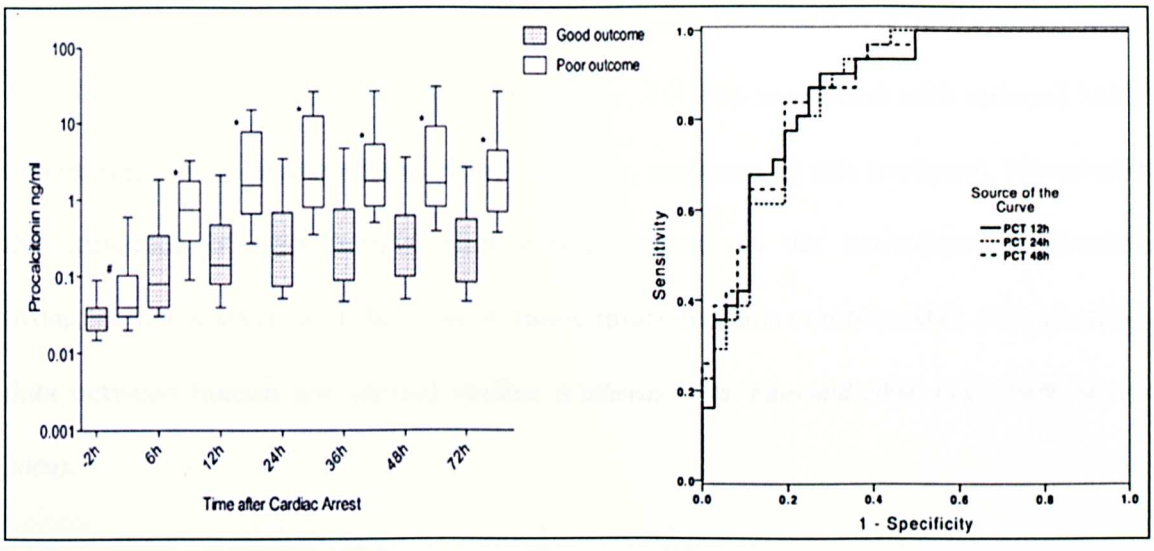


Figure 14. Release pattern for PCT after cardiac arrest in the good and the poor outcome group. The line in the box represent the median, the outer limits of the box represent the inter-quartile range. Outliers are not shown. #p < 0.05, §p < 0.01 and *p < 0.001. Reciever operating characteristic analysis for PCT at 12, 24 and 48 h following cardiac arrest. The area under the curve is 0.88, 0.86 and 0.87 respectively. From *Annborn 2013.*

Matrix metalloproteinases

Other circulating biomarkers, not strictly of cardiac origin, have been evaluated for risk stratification after cardiac arrest. Among them, are the MMPs that play a major role in the turnover extracellular matrix components and cardiac remodeling after ischemia-reperfusion injury. They have been proposed as therapeutic targets in different organs, including the heart *(Dejonckheere 2011)*. The clinical usefulness for risk stratification of MMP-9 has been assessed in 96 cardiac arrest patients *(Turkdogan 2012)*. Circulating levels on admission were significantly higher in patients with failed (93 ng/mL) than with successful CPR (70 ng/mL). In addition, MMP-9 concentration on admission was the sole predictor of early mortality, in multivariable models that included presence of asystole, mean duration of cardiac arrest, out-of-hospital CPR, electrolytes and arterial pH (odds ratio 1.50, p<0.001). At optimal cut-off value, MMP-9 predicted failed CPR with a sensitivity of 88% and a specificity of 98%, suggesting that it might help in risk stratification of patients with cardiac arrest *(Turkdogan 2012)*. Elevation of different MMPs

(MMP-7 and MMP-9) has been confirmed in 51 patients resuscitated form cardiac arrest at 24 hr from ROSC *(Hästbacka 2012)*. In this study, TH was associated with reduced MMP-9 levels, suggesting an attenuation of inflammatory response by this treatment. Nevertheless, the influence of hypothermia after cardiac arrest on the activation of circulating inflammatory markers and their role in tissue injury remains controversial, with conflicting data between human and animal studies *(Callaway 2008, Fairchild 2004, Fries 2009, Meybohm 2009)*.

ECG-derived biomarkers

Guideline statement on "Fibrillation Waveform Analysis to Predict Outcome": There is evidence that VF waveforms change over time. Several retrospective case series, animal studies, and theoretical models suggest that it is possible to predict, with varying reliability, the success of attempted defibrillation by analyzing the VF waveform. However, there are currently no prospective studies that have identified optimal waveforms and/or timing. The value of VF waveform analysis to guide defibrillation management is uncertain (Class IIb, LOE C) *(Deakin 2010, Link 2010)*.

Analyses of ECG features during VF and CPR

The optimal timing of defibrillation can be determined by evaluating the probability of shock outcome. If the shock attempt has a high likelihood of DF success, an electrical shock should be prompted and delivered. Otherwise, unnecessary shocks should be avoided and alternate therapy such as CPR or medications, especially high-quality CC, should be utilized. The search for a reliable indicator of successful DF obtained from the analyses of ECG features has begun more than 20 years ago. The initial approaches to ECG analysis included measurements of VF amplitude *(Weaver 1985)* and frequency *(Brown*

1991). To improve sensitivity and specificity of the ECG predictors for ROSC, more sophisticate methods of VF waveform analyses were introduced and investigated, including wavelet decomposition *(Watson 2004)*, nonlinear dynamics methods *(Callaway 2001)*, and a combination of different ECG parameter analyses *(Eftestøl 2000)*.

Earlier investigations using electrocardiograms focused on *"amplitude or voltage"* of VF wavelets as a predictor of the likelihood of successful DF. *VF voltage*, or signal amplitude, is defined as the maximum peak-to-trough VF amplitude in a given time window of the ECG signal *(Noc 1999)*. Mean VF voltage is the average of VF voltage over the same time interval. It was observed that VF amplitude declines over time and greater amplitudes were associated with correspondingly greater success of DF *(Callaham 1993, Dalzell 1991, Noc 1994, Strohmenger 1996, Stults 1987, Weaver 1985)*. Several studies have shown that this ECG feature reflects vital organ blood flow and specifically myocardial blood flow and energy metabolism *(Brwon 1989, Dalzell 1991, Noc 1999, Strohmenger 1997, Weaver 1985)*. Weaver et al. *(Weaver 1985)* observed that patients in which the VF amplitude was greater than 0.2 mV had a significantly greater likelihood of resuscitation. The observation that DF success rate is higher during the initial period of cardiac arrest, where "coarse VF" with an amplitude greater than 0.2 mV is present, while success of DF is greatly reduced at the stage of "fine VF", has finally evolved to extensive quantitative analysis of ECG waveform. VF voltage appeared not only as a predictor of ROSC but it also affirmed its utility as indicator of VF duration from collapse.

Subsequently, it was realized that other parameters could be computed utilizing fast Fourier transform (FFT) analyses in a selected ECG interval, including VF median frequency, peak power frequency, edge frequency, and spectral flatness measure. The starting point for all these calculations was the *"power spectrum"*, defined as the square of Fourier amplitudes (described in the following paragraphs). Brown et al. *(Brown 1991)*

developed a technique that analyzed VF voltage and VF frequency such to obtain the so called VF *"median frequency" (Brown 1991, Strohmenger 1994).* The median frequency, representing the frequency at which half of the power of the spectrum is above and half below, was calculated by the following equation:

$MF = \Sigma Fi \times Pi / \Sigma Pi$

where Fi is the ith frequency component and Pi the relative power at Fi.

In a porcine model of VF and CPR, a median frequency of more than 9.14 Hz had 100% sensitivity and 92 % specificity in predicting the success of a DF attempt. Frequency analysis of VF wavelets and, specifically, median frequency was also correlated with CPP in animal models as well as human victims and therefore it became the preferred ECG feature to be used as predictor of outcome *(Brown 1989 and 1991 and 1996, Carlisle 1990, Martin 1991, Monsieurs 1998, Stewart 1992, Strohmenger 1996 and 1997).* In addition, this parameter appeared as a more accurate indicator for estimating the duration of untreated VF, compared to the earlier VF amplitude *(Brown 1989 and 1993, Dzwonczyk 1990, Martin 1991).*

Recently, in the pursuit of a more optimal ECG feature prognosticator, several studies focused on the changes and differences of VF waveform features in relationship to the pathophysiology of cardiac arrest. Specifically, investigators focused on the differences between VF as resultant of an ischemic heart disease, which represents the main cause of sudden death, and VF electrically induced, which represents the main experimental model employed in laboratories *(Niemann 2007, Wang 2007b).* Indick et al. *(Indik 2007)* induced VF in swine in which acute myocardial infarction followed legation of the left anterior descending coronary artery (LAD). The study revealed that VF spectral features, such as median, mean, or dominant frequency and bandwidth were significantly reduced compared with those derived from a VF electrically induced. In a porcine model of ischemic induced

cardiac arrest, by acute occlusion of the LAD, we *(Ristagno 2007b and 2011)* have confirmed lower mean VF frequency in comparison to the electrical induced VF (**Figure 15**).

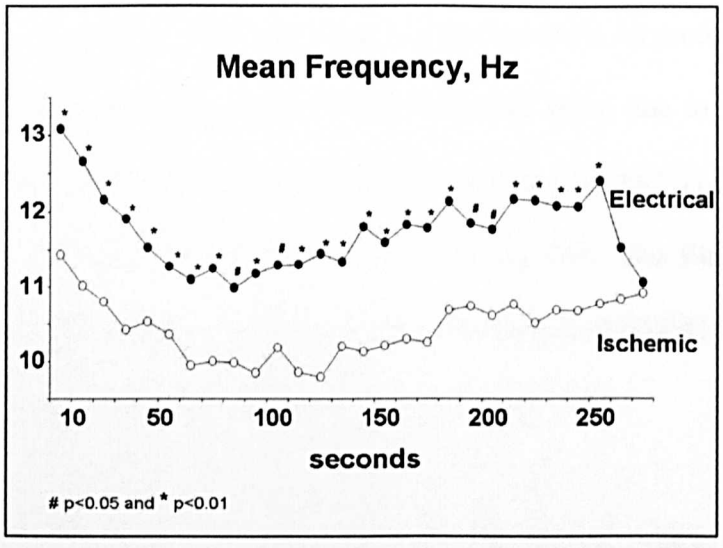


Figure 15. Ventricular fibrillation mean frequency during 5 minutes of untreated VF. Closed circles = VF electrically induced; Open circles = VF ischemically induced.
From *Ristagno 2011.*

Although VF features might be different in relationship to the cause of cardiac arrest, we have observed in the ischemic model of VF that ECG features continuously changed during resuscitative maneuvers. In particular, VF amplitude and mean frequency increased during CC and such increases correlated with successful DF *(Ristagno 2008b and 2008c and 2011)*. These observations provided evidence that ECG predictors of outcome were, at least in part, related to the mechanism by which VF evolved. Of more importance, relations between VF amplitudes, frequencies and success of DF are maintained under the setting of ischemic induced VF, confirming thereby the utility of ECG predictors of outcome. Capability of ECG predictors, i.e. AMSA and its slope, in the presence of AMI was further tested in a porcine model of cardiac arrest with or without AMI *(Indik 2009)*. Untreated VF duration and AMI were independent predictors of ROSC following VF cardiac arrest and AMSA and slope were able to predicted ROSC.

A problem of the majority of the above *predictors* is the corruption of the ECG signal recording during CC. CC, in fact, affects the ECG-extracted parameters, providing

erroneous interventions. The reliability of such predictors, therefore, requires interruption of CC for ECG analyses. This interruption, however, reduces the period of vital myocardial perfusion and has been proven to yield sub-optimal outcome and greater post-resuscitation myocardial dysfunction *(Feneley 1988, Sato 1997)*. At present, several filters and algorithms to reduce and eliminate ECG artefacts and noise due to CC or ambient interferences have been developed and successfully used *(Berger 2007, Li 2008 and 2012, Noc 1999, Pernat 2001, Povoas 2000 and 2002, Strohmenger 1996 and 1997)*. The filtered ECG signals or CPR-artifact-free ECG signals presented better results than the ECG signals with CPR artifacts and with no need for signal preprocessing *(Neurauter 2008)*.

Additionally, the ECG signals recorded from AEDs may also include baseline drifts, powerline interferences, muscle movements, and so on *(Shandilya 2012, Werther 2009 and 2009b)*. A preprocessing step is usually employed to obtain the 'pure' ECG signal before the waveform analysis, including a notch filter to remove alternating current interference at 50-60 Hz, a high-pass filter to remove baseline drifting and CPR artifact, and a low-pass filter to remove the myographic noise *(Amann 2010, Granegger 2011, Irusta 2009, Ruiz 2010, Werther 2012)*. After filtering, the features or characteristics of the VF signal extracted with different digital signal processing methods are then used to predict the probability of DF success based on the established threshold or decision algorithm. To improve sensitivity and specificity of the ECG predictors for DF success and ROSC, more sophisticated methods of VF waveform analyses have recently been introduced and investigated, including wavelet decomposition, nonlinear dynamics methods, and a combination of different ECG parameter analyses.

Different approaches to analyze VF waveform

Different approaches have been employed to analyze VF waveform features: Time domains, Frequency domains, Time-frequency domain methods, Non-linear dynamic, and

combined ones.

Predictors obtained from ***time domain*** describe the characteristics of waveform amplitudes, phases or voltages *(Callaway 2005, Endoh 2011, Joar 2007, Neurauter 2007, Weaver 1985)* and include:

- Peak-to-peak amplitude (PPA), which is defined as the difference between the maximum and minimum recorded VF voltage within a given window.
- The mean amplitude, representing the mean absolute deviation from the mean amplitude of the waveform
- Median slope (MdS) and mean slope (MnS), which represent the average steepness of the waveform, and reflect both the amplitude and frequency information of VF.

Time domain methods are affected by other factors: interference, body size/composition, skin resistance, size and position of electrodes, lead ways, and recording conditions. Additionally, they do not utilize the temporal information to predict the DF outcome.

The ***frequency domain*** features describe the frequency component characteristics of VF waveform. Each frequency component is computed over the clipped ECG segment. Parameters are computed utilizing FFT analyses in a selected ECG interval. FFT consists in splitting a short segment of the VF waveform into small subunits and then expressing each of these as the sum of multiple simpler waves of a given amplitude and frequency, obtaining the so called "power spectrum" (**Figure 16**).

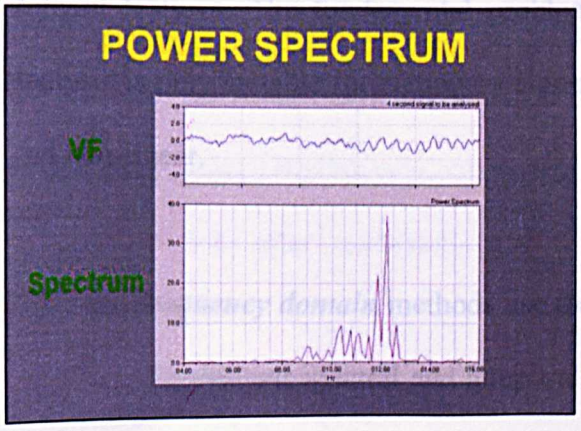


Figure 16. VF fast Fourier transform and its resulting power spectrum.

The calculated features from the FFT of the selected VF window include *(Brown 1996, Eftestøl 2000 and 2001, Endoh 2011, Goto 2003, Gundersen 2008, Hamprecht 2001, Indik 2008, Martin 1991, Neurauter 2007, Stewart 1992, Strohmenger 1997, Watson 2005)*:

- Peak power frequency (PF) or dominant frequency (DF), that is defined as the highest peak in the resulting power spectral density (PSD);
- Energy, calculated as sum of the single power values of the PSD;
- Maximum power, maximum value of PSD;
- Power spectrum area (PSA), which is computed in a similar way to AMSA using PSD instead of amplitude spectral density;
- Centroid power, which is defined as power coordinate of the center of the spectral mass;
- Centroid frequency (CF), which is defined as frequency coordinate of the center of the spectral mass;
- Median frequency (MDF), which is calculated as the mean of all of the contributing frequencies weighted by the power at each frequency;
- Fibrillation power, which is the integral over the fibrillation contribution to the PSD;
- Instantaneous mean frequency;
- AMSA, which is calculated as the sum of contributing frequencies weighted by the absolute values of the FFT of the VF signal, describes the amplitude-weighted mean frequency.

Frequency domain features are robust and less affected by external factors than the time-domain features. The fundamental problem of frequency domain methods is that FFT analysis is only suitable for stationary signals whereas the ECG signals are non-stationary and non-linear.

The ***time-frequency domain*** methods use the continuous or discrete wavelet transform that provides concomitant spectral and temporal information, allowing a local scale-dependent

spectral analysis of signal features. They include *(Box 2008, Martin 1991, Watson 2004 and 2005)*: wavelet-based PF; energy; wavelet-based MNF; spectral flatness; entropy, i.e. the cardioversion outcome prediction (COP) (wavelet-entropy marker), which is used as a metric of the temporal behavior of the signal.

Earlier research has confirmed that VF is a complex non-linear pattern formed by drifting spiral waves of electrical activity (vortices and rotors) that travel across the myocardium and subsequently break down. The ***non-linear dynamic*** methods, however, are sensitive to the noise and interference. There are several reported non-linear features to predict DF success *(Callaway 2001, Jagric 2007, Jalife 1996, Lin 2010, Panfilov 1998, Podbregar 2003, Rodriguez 2009, Sherman 2008)*:

- The scaling exponent (ScE), which is an estimate of the fractal self-similarity dimension;
- Hurst exponent, which is used as a measure of long term memory of time series;
- Irregularity, which is a direct indicator of chaotic behavior;
- the logarithm of the absolute correlations (LAC), which quantifies how individual parts of a signal are self-similar at different points along its length. It provides information regarding the duration of VF by measuring the roughness of the VF waveform;
- detrended fluctuation analysis (DFA), which determines the statistical self-affinity of VF waveform.

Beside the different ECG predictors that can be used, there is also no consistent definition for DF success. Two definitions are commonly adopted for successful DF: (1) the presence of an organized rhythm at 5 sec after the DF attempt *(Koster 2006)*; (2) the presence of a sustained organized rhythm originating within a min after the DF attempt *(Watson 2005)*; (3), survival till 6 hr after resuscitation or discharge from the hospital (the most rigorous definition).

Among the numerous studies available on different predictors of DF success (**Table 6**), sensitivity for this approach ranges from 54% to 100% with a specificity ranging from 23% to 98%. The majority of studies, however, included only a few patients (median of 86 patients). Only two studies had more than 200 patients.

Table 6. DF outcome prediction with different ECG predictors (clinical studies).

Study	VF feature	Patients (no.)	Sensitivity	Specificity	Outcome
Dalzell 1991	PPA	70	NA	NA	ROSC
Martin 1991	MDF	7	NA	NA	ROSC
Brown 1996	Combination of PF and CF	55	100	47	ROSC
Strohmenger 1997	PPA, MDF	26	100	25	ROSC
Eftestol 2000	Combination of CF and PF	156	92	42	ROSC
Strohmenger 2001	DF	89	92	42	ROSC
Eftestol 2001	Combination of CF and Energy	156	91	36	ROSC
Hamprecht 2001	DmF	54	59	52	ROSC
Podbregar 2003	Combination of PPA, total energy of PSD and Hurst exponent	47	100	97	ROSC
Jekova 2004	Energy (2-7 Hz)	NA (more than 700 trace data set)	62	80	ROSC
Young 2004	AMSA	46	91	94	ROSC
Watson 2004	Entropy	NA (868 trace data set)	91	60	ROSC
Watson 2005-2006	COP	110	97	63	ROSC
Neurauter 2007	MdS	197	95	53	ROSC
Box 2008	COP	54	100	60	ROSC
Ristagno 2008b	AMSA	90	91	97	ROSC
Neurauter 2008	MdS (10-22Hz)	192	95	50	ROSC
Lin 2010	DFA (DFAα2)	155	61	63	ROSC
Endoh 2011	CF	152	77	63	ROSC
Shanmugasundarama 2012	Slope	44	83	70	ROSC
Nakagawa 2012	AMSA	83	94	59	ROSC
Weaver 1985	PPA	394	97	23	Survival
Monsieurs 1998	Survival index	100	70	70	Survival
Goto 2003	DF	47	77	90	Survival
Callaham 1993	PPA	265	54	98	Survival
Callaway 2001	ScE	75	NA	NA	Survival

NA, not available.

The potential benefit of a DF guided by a real time VF waveform analysis, if successful, would allow for optimization of the timing for shock delivery, such to maximize DF success and minimize the number of unsuccessful shocks. The risk of this intervention is minimal, as the ECG signal which would be analyzed is already readily available. Currently the following gaps are present in the studies:

- Data on factors responsible for changes in VF waveform, (i.e., time of arrest, quality of CPR, drug administration, concomitant AMI)
- Determination of which VF waveform parameter is the most accurate
- Influence of TH and patient temperature on VF waveform

Evolution of Amplitude spectrum area (AMSA)

The need for ECG analyses and prediction for successful DF escalated following the introduction of AEDs. Initial efforts at the Institute of Critical Care Medicine focused on the ECG indicator widely investigated at that time and specifically evaluated the possibility of VF amplitude to predict resuscitability in a rodent model of cardiac arrest and CPR. Increases in CPP during CCs were associated with concomitant increases in VF voltage and greater VF voltages were observed in successfully resuscitated animals. Moreover, greater VF voltages after initiation of cardiac resuscitation were associated with increases in myocardial creatine phosphate, and significant decreases in lactate content. Accordingly, increases in VF voltage during cardiac resuscitation reflected increases in myocardial perfusion and favorable changes in myocardial energy metabolism with consequent greater success of CPR *(Noc 1994)*. In a porcine model of cardiac arrest and CPR, successfully resuscitated animals had significantly greater CPP, dominant VF amplitude, mean VF amplitude, and dominant VF frequency. No animals were resuscitated if CPP was <8 mm Hg, dominant amplitude <0.48 mV, mean amplitude <0.25 mV, or dominant frequency <9.9 Hz, independently of the duration of untreated VF. However, DF attempts uniformly

failed when mean amplitude was below the threshold level even though dominant frequency would have predicted otherwise. Efforts to obtain a better predictor led to the "defibrillator predictor" in which mean amplitude and dominant frequency were combined. Utilizing stepwise multiple regression analysis, the investigators identified a single numerical score which was established as a DF predictor and wss represented by the following equation *(Noc 1999)*:

*Defibrillator Predictor=3.60-4.85 * mean VF amplitude-0.06 * VF dominant frequency.*

This predictor served as an objective noninvasive measure on par with that of CPP for predicting the success of DF. Defibrillations were unsuccessful if the combination of mean amplitude and dominant frequency did not exceed a specific threshold value. Unfortunately, PPV was still suboptimal, being 20%. Drs. Pernat and Povoas *(Pernat 2001, Povoas 2000 and 2002)*, continuing the earlier studies, finally introduced the new DF outcome predictor, namely AMSA. This ECG-derived parameter was obtained from conventional scalar limb ECG leads, continuously monitored during uninterrupted CC. The electrocardiographic signal was sampled and recorded at a frequency of 300 Hz. The amplitude spectrum was obtained by FFT of the ECG scalar signal (**Figure 17**). AMSA was then calculated from the resulting amplitude frequency spectrum according to the following equation:

$AMSA = \Sigma Ai \times Fi$

where Ai is the amplitude at the ith frequency Fi.

To minimize low-frequency artefacts produced by CC and to exclude the electrical interference of ambient noise at frequencies greater than 48 Hz, the analyses were performed between the frequencies of 4 and 48 Hz.

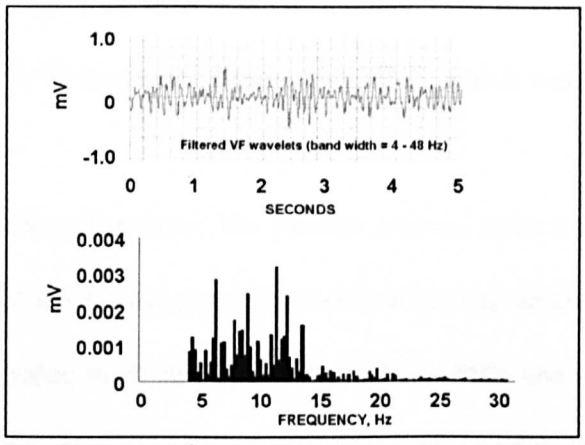


Figure 17. A representative example of the amplitude frequency relationship and the area under the curve that defines AMSA.

In an initial study, Dr. Povoas *(Povoas 2000)* investigated CPP, obtained from arterial and right atrial pressures and VF mean amplitude, MNF, and AMSA, obtained from ECG recordings in 55 domestic pigs during CPR. Form these measurements *threshold* values for ROSC were obtained and subsequently validated in another 10 animals. CPP and mean amplitude each had PPVs of 100% but NPVs of only 44% and 22%, respectively. MNF predicted successful DF with a PPV of 75% but a NPV of only 30%. AMSA yielded a better combination of positive and negative predictive values of 86% and 85%, respectively. Among the exciting and impressive results obtained with this new ECG parameter, the high NPV was of special note since the investigators realized that AMSA was able to minimize repetitive and ineffective electrical shocks during CPR.

During the same period, Dr. Pernat confirmed these results, demonstrating the capability of AMSA to optimize the timing of DF *(Pernat 2001)*. In a porcine model of cardiac arrest and resuscitation, AMSA was highly correlated with CPP levels during CPR and AMSA, similarly to CPP, was significantly greater in animals that were resuscitated compared to those that were not. In no instances a perfusing rhythm was restored when AMSA was < 21.0 mV-Hz. The AMSA value of 21 mV-Hz predicted restoration of perfusing rhythm with sensitivity and specificity above 90%. The NPV of AMSA was 95% and statistically equivalent to that of CPP, mean amplitude, and MNF. However, the PPV, was greatly

improved with AMSA, namely 78% in contrast to the lower predictive values yielded by CPP, mean amplitude, and MNF, which were below 40%.

One year later, Dr. Povoas *(Povoas 2002)* further investigated the real time application of AMSA in a porcine model of cardiac arrest. The investigators confirmed that an AMSA value of 21 mV-Hz had a NPV of 96% and a PPV of 78%. An AMSA value of 21 mV-Hz or greater predicted restoration of a perfusing rhythm in 7 of 8 instances and AMSA of 20 mV-Hz or less correctly predicted failure of electrical resuscitation in 24 of 26 instances. The progressive increases in AMSA observed before successful resuscitation further demonstrated that AMSA had the potential of providing an objective guide allowing for better quality control of CPR. Failure to increase AMSA values to near threshold levels prognosticated failure of defibrillation.

These initial empirical trials identified AMSA as a good predictor for guiding the DF attempt. Subsequent *validation* studies confirmed that AMSA had impressively higher specificity and PPV compared with the other predictors, maintaining sensitivity and NPV comparable to CPP, mean amplitude, and MNF. More importantly, AMSA was not invalidated by artefacts resulting from CC fulfilling the goal of a predictor that would allow for uninterrupted CC during ECG analyses. AMSA was well correlated with CPP, which is widely recognized as the gold standard for predicting the success of defibrillation. Yet, CPP was robust only for negative prediction. It is the specificity and the PPV, which are assured by AMSA that are more likely to minimize the adverse effects of repetitive high energy shocks during CPR and the resulting post-resuscitation myocardial dysfunction.

In more than 65% of the cardiac arrest events, the usual cause is an underlying AMI *(Podrid 2005)*. Accordingly, myocardial ischemia and reperfusion have been involved in the

triggering of malignant ventricular dysrhythmias *(Ouyang 1981, Qin 2002)* and both the duration and the severity of myocardial ischemia play important roles in developing myocardial cell damage *(Reimer 1979)*. Indeed, we have earlier described that AMSA was superior to CPP as indicator of return to a perfusing rhythm following DF, under condition of partial occlusion of the LAD *(Ristagno 2007b)*. In a porcine model of cardiac arrest and resuscitation, a partial occlusion of the LAD, which was approximately 75% of the internal lumen, was maintained during CPR. During CC, CPP increased and exceeded threshold value for successful resuscitation. AMSA however, was significantly lower in the animals in which the partial occlusion of the LAD was maintained during CPR (**Figure 18**). This was reflected by the greater number of electrical shocks required to terminate VF with lesser success of resuscitation. CPP is in fact, an indirect indicator of myocardial flow produced by CC and represents a gradient pressure between aorta and right atrium. This gradient can be maintained even in the presence of occlusion of the coronary tree. AMSA, which is instead related to myocardial blood flow and metabolism, has been shown to substantially decrease when the myocardial perfusion is truly reduced.

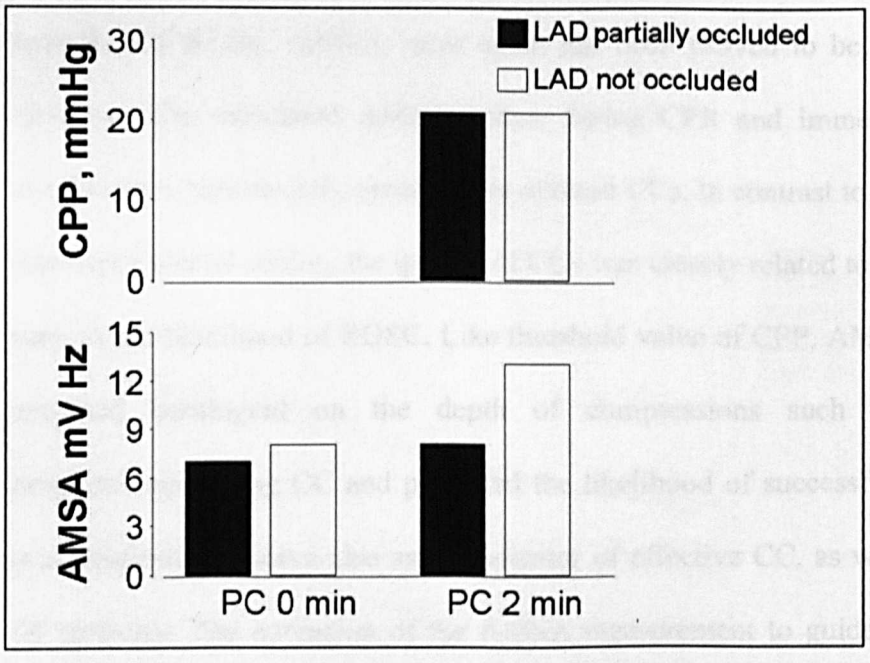


Figure 18. Coronary perfusion pressure (CPP) and amplitude spectrum area (AMSA) at onset of precordial compressions (PC 0 min) and two minutes later (PC 2 min). $p < 0.01$ for AMSA at PC 0 min vs AMSA at PC 2 min in the group with LAD partially occluded. From *Ristagno 2011.*

Accordingly, the quality of CC is a major issue for CPR success *(Gallagher 1995, Van Hoeyweghen 1993, Wik 1994)*. Effectiveness of CC relates to compression depth, compression rate and duty cycle, and complete chest recoil during the releasing phase *(Brown 2006, Field 2010, Nolan 2010, Travers 2010)*. Outcome may be improved by assuring adequate depth of compression in addition to more optimal rates of compression *(Abella 2005, Wik 2005)*. Since CC is usually performed without feedback and relatively small changes in the depth of compression profoundly alter hemodynamic effectiveness and outcome, there is an increasingly recognized need for a monitor of effectiveness of CC *(Aase 2002, Baubin 1999, Noordergraaf 2006, Travers 2010)*. Recently, we have investigated the possibility of assessing the quality of CPR, and especially of CC depth, utilizing AMSA, which has the important advantage of being non-invasive and calculable from the universally available ECG, as part of the current practices of advanced life support. In a porcine model of VF and CPR, animals were randomized to either optimal or suboptimal CC depth after onset of VF. Optimal depth of mechanical compression was defined as a decrease of 25% in anterior posterior diameter of the chest during compression. Suboptimal compression was defined as a decrease of 17.5% in anterior posterior diameter. All animals had ROSC after optimal compressions. This contrasted with suboptimal compressions after which none of the animals had ROSC. AMSA, once again has been proven to be, like CPP, predictive of outcome. The calculated AMSA values during CPR and immediately prior to the DF attempt were significantly greater after optimal CCs, in contrast to the sub-optimal ones. In that experimental setting, the quality of CCs was closely related to the AMSA value and in turn, to the likelihood of ROSC. Like threshold value of CPP, AMSA threshold value was achieved contingent on the depth of compressions such that AMSA increased progressively during CC and predicted the likelihood of successful DF. AMSA therefore was confirmed to serve also as an indicator of effective CC, as well as a tool for guiding DF delivery. The extension of the AMSA measurement to guide the quality of CC was explained as the capability to restore the electrical robustness of the myocardium through

restoring threshold level of coronary blood flow. When AMSA is of insufficient magnitude, the rescuer is prompted to push harder and perhaps to push faster.

Beside the quality of CC, AMSA might also be useful for retrieving the duration of untreated VF in the case of unwitnessed cardiac arrest *(Niemann 1992, Ristagno 2011)*. In nine domestic male pigs, VF was induced and left untreated for 15 min *(Ristagno 2011)*. AMSA, more than VF amplitude and frequency, was highly correlated with the downtime of VF and decreased over time, as shown in **Figure 19**. Significantly lower AMSA was observed after 3 min of untreated VF. Following the 4th min of VF, AMSA values decreased more rapidly. This additional use of AMSA therefore may provide guidance to the best initial intervention rescuer should adopt upon arrival at the cardiac arrest scene, i.e. DF or CC first.

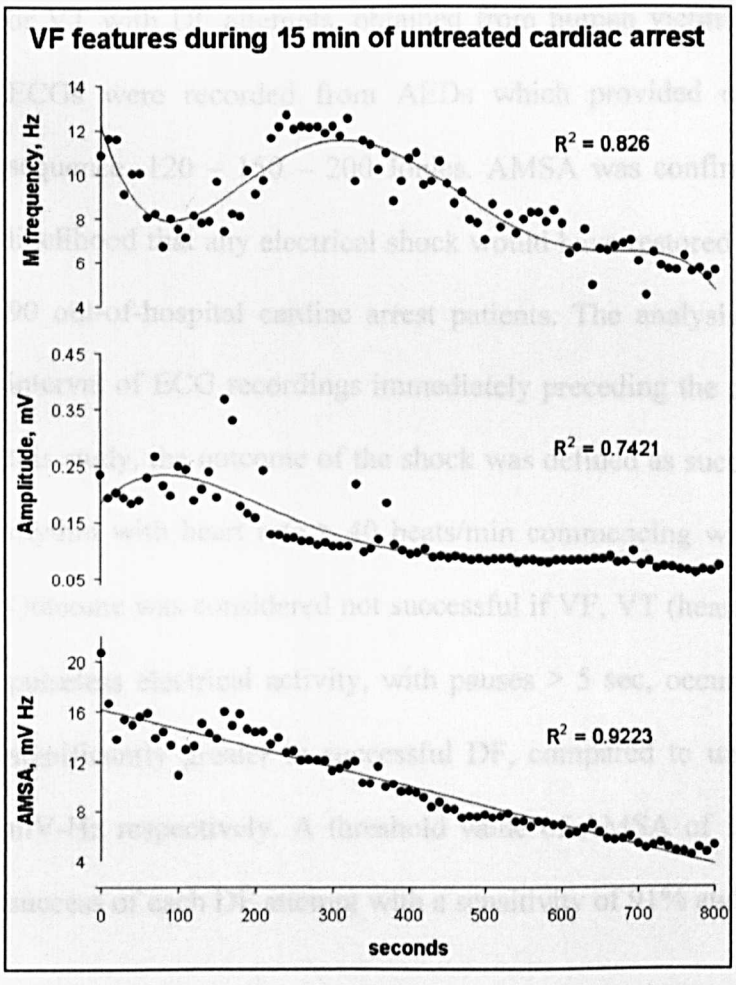


Figure 19. Mean (M) frequency, amplitude and AMSA during 15 minutes of untreated ventricular fibrillation.
From *Ristagno 2011.*

Applicability of AMSA to the clinical scenario

The subsequent step in the evolution of AMSA as an indicator of effectiveness of intervention and to guide DF was the confirmation of its efficacy in clinical settings. The first confirmation of the capability of AMSA to predict success of defibrillation and ROSC in a clinical scenario was reported by Young et al. *(Young 2004)* in the late 2004. This study was a retrospective analysis of ECG traces, representing lead 2 equivalent recordings, on 108 DF attempts on 46 victims of cardiac arrest due to VF during out-of-hospital cardiac arrest. An AMSA value of 13 mV-Hz predicted successful DF, with a sensitivity of 91% and a specificity of 94%. This data represented the first evidence of the capability to extend the predictive value of AMSA to human patients.

In 2008, Ristagno et al. *(Ristagno 2008b)* analyzed a new database including episodes of VF or VT with DF attempts, obtained from human victims of out-of-hospital cardiac arrest. ECGs were recorded from AEDs which provided escalating biphasic shocks in the sequence, 120 – 150 – 200 Joules. AMSA was confirmed as a valid tool to predict the likelihood that any electrical shock would have restored a perfusing rhythm during CPR in 90 out-of-hospital cardiac arrest patients. The analysis was performed on a 4.1 second interval of ECG recordings immediately preceding the delivery of the DF. For purpose of this study, the outcome of the shock was defined as successful if DF restored an organized rhythm with heart rate $\geq$ 40 beats/min commencing within the 1 min post shock period. Outcome was considered not successful if VF, VT (heart rate > 150 beats/min), asystole or pulseless electrical activity, with pauses > 5 sec, occurred after DF. AMSA values were significantly greater in successful DF, compared to unsuccessful one, 16 mV-Hz and 7 mV-Hz respectively. A threshold value of AMSA of 12 mV-Hz was able to predict the success of each DF attempt with a sensitivity of 91% and a specificity of 97%.

The PPV, which refers to the proportion of the shocks that were correctly predicted to restore a perfusing rhythm, was 95%. The NPV, which instead refers to the proportion of the shocks that were predicted to fail and actually failed to restore a perfusing rhythm, was 97%. The results of this study were consistent with the previous retrospective analysis *(Young 2004)*. Of particular interest was the fact that although different models of defibrillators were employed in the two studies, the results were consistent. This was therefore a further confirmation that AMSA represented an excellent predictor of DF success, and this capability was independent from the defibrillatory energies and waveforms utilized. Finally, in a recent study including 267 CPR sequences from 77 victims of out of hospital cardiac arrest, Dr. Eftestøl and colleagues *(Eftestøl 2004)* confirmed AMSA as one of the most powerful predictor of success of defibrillation.

AIM OF THE STUDIES AND HYPOTHESES

The present thesis includes both experimental and clinical studies directed to discover and/or validate experimentally and clinically new circulating and ECG-derived biomarkers predictive of outcome of cardiac arrest.

- ***CIRCULATING BIOMARKERS:***

- **STUDY 1: Discovery of new circulating biomarkers with the aid of untargeted metabolomics in a rat model of cardiac arrest**

With the aim to discover new circulating biomarkers better able to explain the physiopathology of the post-cardiac arrest syndrome and to predict outcome of cardiac arrest, we used an untargeted metabolomics approach. Metabolomics is the global study of metabolite changes in a biological system. The metabolome can be viewed as the biochemical consequence of such changes, the real effectors of a phenotype *(Brown 2009, Kell 2005, Old 2005, Zhang 2006)*. The comprehensive quantitative assessment of plasma metabolites and in particular of post-resuscitation metabolite differences in plasma may provide important information about the cellular metabolism response to CPR. The aim of this study was to examine post-resuscitation plasma metabolites in a rat model of cardiac arrest and resuscitation to identify perturbation in circulating metabolites and thus potential mechanisms accounting for outcome. We hypothesized that extensive characterization of the largest possible number of metabolites from relevant or potentially affected metabolic pathways might help in identifying mechanisms explaining the outcome of cardiac arrest and CPR and might serve as early prognosticator biomarkers.

- **STUDY 2: Validation of KP activation after cardiac arrest with experimental models in rats and pigs and in a small cohort of cardiac arrest patients**

Results from study 1 *(Brunelli 2013)* were able to identify alterations in a major route of the TRP catabolism, namely kynurenine pathway, shown in **Figure 20**. KP is mainly activated

upon inflammatory stimulation and is implicated in the pathogenesis of numerous central nervous system disorders, as well as in sepsis development and profound hypotension during septic shock *(Changsirivathanathamrong 2011, Wilson 2012)*. KP activation has been also described in various clinical conditions, including infection, autoimmune syndromes, malignancies, depression, and pregnancy *(Huttunen 2010, Maes 2011)*.

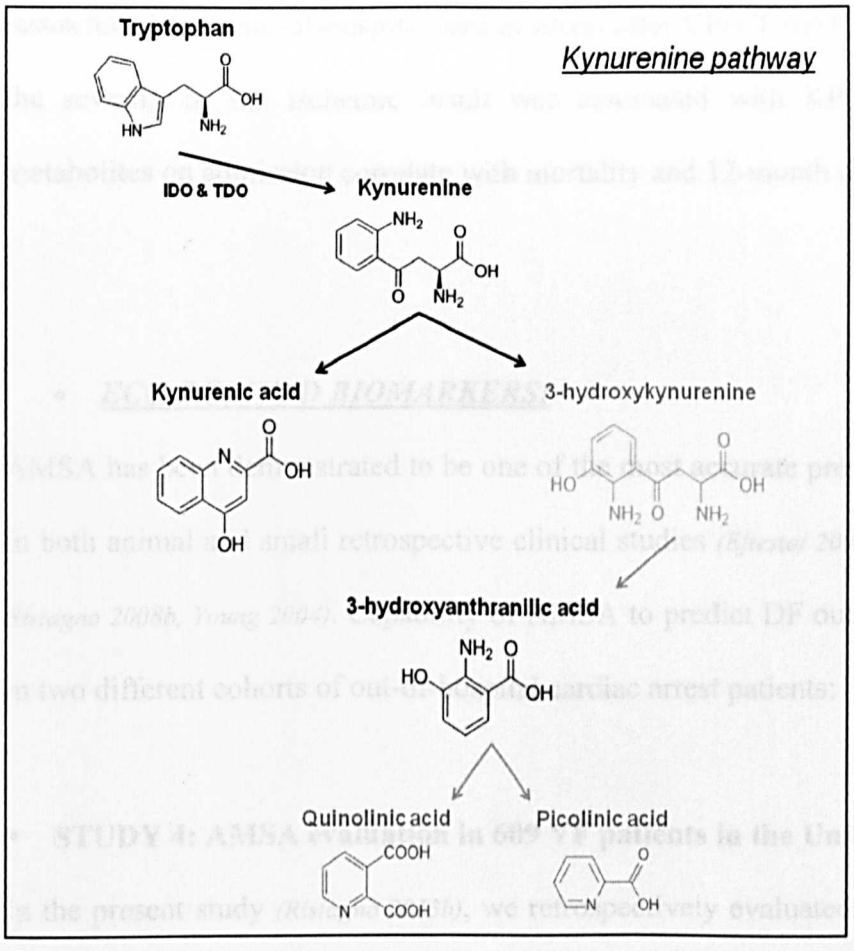


Figure 20.
Tryptophan degradation through the kynurenine pathway. In black are the metabolites assayed in the present study.
TDO, tryptophan 2,3-dioxygenase;
IDO, indoleamine 2,3-dioxygenase.
From *Ristagno 2013.*

The following studies, therefore, aimed to investigate KP activation after cardiac arrest and its relationship with the severity of post-cardiac arrest syndrome, by a fully translational approach *(Ristagno 2013)*. More specifically, KP was assessed during the initial hours and days following resuscitation from cardiac arrest in rats, pigs, and in a small cohort of patients. Based on the above findings, specific KP metabolites were then targeted, using LC multiple reaction monitoring (MRM)-mass spectrometry. We hypothesized that the KP

would be activated following cardiac arrest and this activation would be associated with the severity of post-resuscitation organ dysfunctions and outcome.

- **STUDY 3: Validation of KP activation after cardiac arrest in a large cohort of out of hospital cardiac arrest patients**

In the present observational cohort study we examined the KP in critically ill patients resuscitated from out-of-hospital cardiac arrest after VF/VT (n=155). We hypothesized that the severity of the ischemic insult was associated with KP activation and that KP metabolites on admission correlate with mortality and 12-month outcome.

- ***ECG-DERIVED BIOMARKERS:***

AMSA has been demonstrated to be one of the most accurate predictors for successful DF, in both animal and small retrospective clinical studies *(Eftestøl 2004, Pernat 2001, Povoas 2002, Ristagno 2008b, Young 2004)*. Capability of AMSA to predict DF outcome has been evaluated in two different cohorts of out-of-hospital cardiac arrest patients:

- **STUDY 4: AMSA evaluation in 609 VF patients in the United States**

In the present study *(Ristagno 2013b)*, we retrospectively evaluated the capability of AMSA to predict DF success in a large database of out-of-hospital VFs. We hypothesized that AMSA, derived from conventional AED pads, would be an useful indicator to predict DF success and guide CPR interventions. We further hypothesized that AMSA could serve as a monitor of CC quality.

- **STUDY 5: AMSA evaluation in 1.617 VF patients in Lombardia region, Italy**

We evaluated the capability of AMSA to predict DF outcome in out-of-hospital cardiac arrest, in a Region of Northern Italy. We hypothesized that threshold values of AMSA

could be identified to be used as a guide for CPR intervention and as a predictor of DF outcome. More specifically, AMSA thresholds for DF success and failure were calculated from a derivation cohort of 1.050 VF patients receiving DF attempts; these thresholds were then validated in an additional cohort of 567 VF patients. Effects of different factors potentially affecting VF waveform have been also investigated (i.e., time of arrest, drug administration, co-morbidities, etc.). Furthermore, relationship between AMSA and long-term outcome, i.e. hospital discharge and 1 year survival, has been evaluated. Finally, comparison between AMSA and classical VF waveform parameters has been performed.

MATERIALS AND METHODS

Procedures involving animals and their care were in compliance with national (D.L. n. 116, G.U., suppl. 40, 18 February 1992, Circolare no. 8, G.U., 14 Luglio 1994) and international laws and policies (EEC Council Directive 86/609, OJL 358, 1, December 12, 1987; Guide for the Care and Use of Laboratory Animals, US National Research Council, 1996). Approvals of the studies were obtained by the local institutional review board committee and governmental institution *(Fries 2012, Ristagno 2013b)*.

- ***CIRCULATING BIOMARKERS:***

- **STUDY 1: Discovery of new circulating biomarkers with the aid of untargeted metabolomics in a rat model of cardiac arrest**

Standards and chemicals for metabolomic analysis

High-performance liquid chromatography (HPLC)-grade acetonitrile, ammonium acetate (>99% dry matter) and formic acid (98%) were purchased from Fluka (Buchs, Switzerland). HPLC grade MilliQ water was obtained with a MILLI-RO PLUS 90 apparatus (Millipore, Molsheim, France). Analytical standards L-tryptophan, L-kynurenine, KYNA, and 3-HAA were from Sigma-Aldrich (Italy). Deuterated standards D8-L-tryptophan and D6-kynurenic were from CDN Isotopes (Chemical Research 2000 S.r.l, Italy). D4-L-kynurenine and D2-3-hydroxyanthranilic acid were from Buchem BV (Netherlands). Individual stock solutions were prepared in MilliQ water and stored at -20°C. Working solutions containing all the metabolites and their internal standards were prepared freshly before analysis.

Rat model of cardiac arrest

An established rat model of cardiac arrest and CPR was used (for more details, refer to STUDY 2). Twelve male Sprague–Dawley rats weighing 450 ± 30 g were anesthetized with pentobarbital, endotracheally intubated and surgically instrumented for femoral artery cannulation for blood withdrawal. VF was induced in 6 rats by delivering up to 4 mA AC current into the right ventricle. CPR, including mechanical CC, ventilation with oxygen, and epinephrine (0.02 mg/kg), was then started and continued for a 6 min before DF. CC was maintained at a rate of 200/min with equal compression–relaxation and ventilation at 50/min. Resuscitation was attempted with up to three two-joule counter-shocks. Animals were considered successfully resuscitated if supra-ventricular rhythm returned with mean aortic pressure above 50 mmHg. Two hr after resuscitation, blood was withdrawn and animals were euthanized. Blood was collected into ethylenediaminetetraacetic acid (EDTA) tubes and centrifuged for 10 min at 2,000×g at room temperature. Plasma samples from each animal were immediately stored at −80 °C. Six other rats were not subjected to cardiac arrest and served as controls (CTR). Body temperature was held at 37 ± 0.5 °C throughout the experiment.

Metabolomic profiling by LTQ-Orbitrap mass spectrometry

Discovery pilot metabolomics analysis began with an unbiased search for plasma analytes linked to post-resuscitation using two experimental groups from the rat model. The control group (CTR, n=3) was selected from rats not subjected to cardiac arrest, while the case group was selected from rats subjected to cardiac arrest and CPR and sacrificed 2 hr after resuscitation (CA/CPR2, n=3, early post-resuscitation phase). To reduce biological variation that might mask important changes in metabolite abundances, we prepared metabolite samples by pooling plasma (5 μL/animal) from individual rats in the experimental groups. Every

specimen made an equal contribution to the pool and two composite groups (CTR and CA/CPR2) were then created. Metabolites were extracted by adding four volumes of cold methanol to the plasma sample; samples were vortexed and incubated at -20°C for 1 hr. They were then centrifuged 10 min at 14.000 x g, and the supernatant (rich in small-molecules analytes) was collected, dried in a SpeedVac and resuspended in 20 µL of 0.1% formic acid. A portion (2 µL) of metabolite extract from the CTR and CA/CPR2 groups was directly analyzed by liquid chromatography (LC) – mass spectrometry (MS)/MS, using an LTQ Orbitrap XL™ (Thermo Scientific, Waltham, MA, US), interfaced with a 1200 series capillary pump (Agilent, Santa Clara, CA, US). The MS instrument was operated in positive (POS) and negative (NEG) ionization modes. Analyses were run in triplicate. Metabolites were separated on an Agilent Technologies Zorbax C18 SB column (150 x 0.5 mm ID, particle size 5 µm). Flow rate 10 µL/min with mobile phases: water containing 0.1% formic acid (A) and acetonitrile (B) for the positive ion. For negative ion mode, 2 mM of ammonium acetate was substituted for the 0.1% formic acid. The gradient consisted of 5% B for 5 min, followed by a linear gradient to 95% B over 45 min, hold at 95% B for 5 min, and re-equilibration at 5% for 2 min.

MS conditions were: source DESI Omni Spray (Prosolia, Indianapolis, IN) used in nanospray mode with positive and negative ion modes; ion spray voltage 2100 V; capillary temperature 220°C; capillary voltage, 42 V. MS spectra (m/z 100-1000) were acquired in the Orbitrap analyzer at 60,000 resolution, in parallel with the low-resolution MS/MS scans of the four most abundant precursor ions being acquired in the LTQ. The lock-mass option was used to obtain the most accurate mass measurements in MS mode. The polydimethylcyclosoloxane ion generated in the electrospray process from the ambient air (protonated (Si(CH3)2O)6, m/z 445.120025) was used for internal recalibration in real time. MS/MS analysis was done in data-dependent mode (DTA) using Xcalibur software (Thermo Scientific, Waltham, MA, US)

with target ions previously selected for the MS/MS dynamically excluded for 30 sec. To ensure the stability and repeatability of the LC-MS systems, ten runs of pooled samples were done on the system before the sample run sequence. Samples were run in an order that alternated the CTR and CA/CPR2 groups to reduce any systematic error associated with instrumental drift.

Untargeted metabolomics data processing and statistical analysis

All LC-MS files were analyzed using the MS label free differential analysis software SIEVE v1.3 (ThermoFisher, Cambridge, MA, US). SIEVE was run on all the LC-MS full-scan chromatograms using the small molecule setting. The chromatograms were time-aligned, referencing the CA/CPR2 sample acquired in the middle of the sequence. The framing parameters were set at 0.01 Da for the m/z window and 0-35 min for the retention time (RT) window; 500,000 was used as the intensity threshold. Prior to performing any statistical analysis, an additional filtering criteria was applied to include in the dataset only frames with an intensity coefficient of variation (CV%) <10. The preprocessed results were then fed into the SIMCA-P 13 (Umetrics, Umea, Sweden) platform for multivariate analysis. Principal component analysis (PCA) was performed on intensity data, preprocessed using the Pareto scaling, to examine cluster and outliers within the observations. All analyses were performed on data from both ion modes separately. Univariate analysis was performed using a 2-tailed Welch t-test ($p<0.01$; Prism v. 5.0, GraphPad Software Inc, USA) to identify metabolites presenting intensities significantly different in the two experimental groups.

Identification of plasma metabolites

For metabolite identification, the frame m/z values were used for batch searches on the METLIN database (http://metlin.scrpss.edu) and Human Metabolome Database (HMDB,

http://www.hmdb.ca/). Both sites allow the user to search by ionization mode, either positive or negative. Accurate mass data and isotopic distribution for the precursor and product ion were compared to spectral data of the reference compounds in the databases. Definite identifications were reported only for metabolites with accurate mass match <5 ppm.

Mapping metabolic pathways

For biological interpretation of the metabolite dataset by our untargeted strategy, we mapped the identified metabolites to the KEGG pathway database (Kyoto Encyclopedia of Genes and Genomes; (www.genome.jp/kegg/), using MetaboAnalyst 2.0, a comprehensive online tool suite for metabolomic data analysis and interpretation (www.metaboanalyst.ca). Metabolite sets were analyzed to identify biologically meaningful patterns that were significantly enriched in our metabolomic data. The Over Representation Analysis (ORA) algorithm was applied and the hypergeometric test was used to see whether a particular metabolite set was represented more than expected by chance in the given compounds list. Then the Pathway Analysis Module was used to combine the enrichment analysis results with the pathway topology analysis (centrality measures to estimate node importance) to identify the most important pathways involved in early CPR *(Xia 2011)*.

Absolute quantification of plasma TRP metabolites by LC-MRM coupled with isotope-dilution mass spectrometry

The absolute quantification of TRP metabolites was performed in plasma obtained from a total of 6 rats subjected to cardiac arrest and CPR and sacrificed 2 hr after resuscitation (by adding three additional experiments to the earlier ones), as previously described. A control group (CTR, n=6) was selected from rats not subjected to cardiac arrest (by adding three additional controls to the earlier ones). Plasma (20 μL) from each animal in each experimental group was

spiked with 10 µM of deuterated standards (tryptophan-D8, L-kynurenine-D4, kynurenic acid-D5, 3-hydroxyanthranilic acid-D2). Spiked plasma samples were then deproteinized by mixing with four volume of cold methanol, vortexed, and incubated at -20°C for 1 hr. Samples were centrifuged 10 min at 14.000 x g, the supernatant was collected, and the centrifugation was repeated. The supernatant was dried in a SpeedVac and resuspended in 20 microL of 0.1% formic acid. Ten µL of supernatant were analyzed directly by LC-MS/MS with the Agilent 1200 series system for LC. Separation was with a Synergy 4u Fusion-RP 80A column (50x2.00 mm, Phenomenex) using as mobile phase A 0.1% formic acid in water and mobile phase B 100% acetonitrile at a flow rate of 0.2 mL/min. Elution started with 99% of A and 1% of B, followed by a13-min linear gradient to 99% of B, a 2-min isocratic elution and a 1-min linear gradient to 99% of A, which was maintained for 8 min to equilibrate the column. The mass spectrometric analysis was done using an Agilent 6410 triple quadrupole mass spectrometer (Agilent Technologies) in positive ion mode for all the metabolites. Typical chromatograms from the analysis of rat plasma are presented in **Figure 20.** Instrumental conditions optimized for each compound are summarized in **supplementary Table 1** (Appendix). Quantitative analyses were processed with MassHunter workstation quantitative analysis software v B.01.04 (Agilent Technologies).

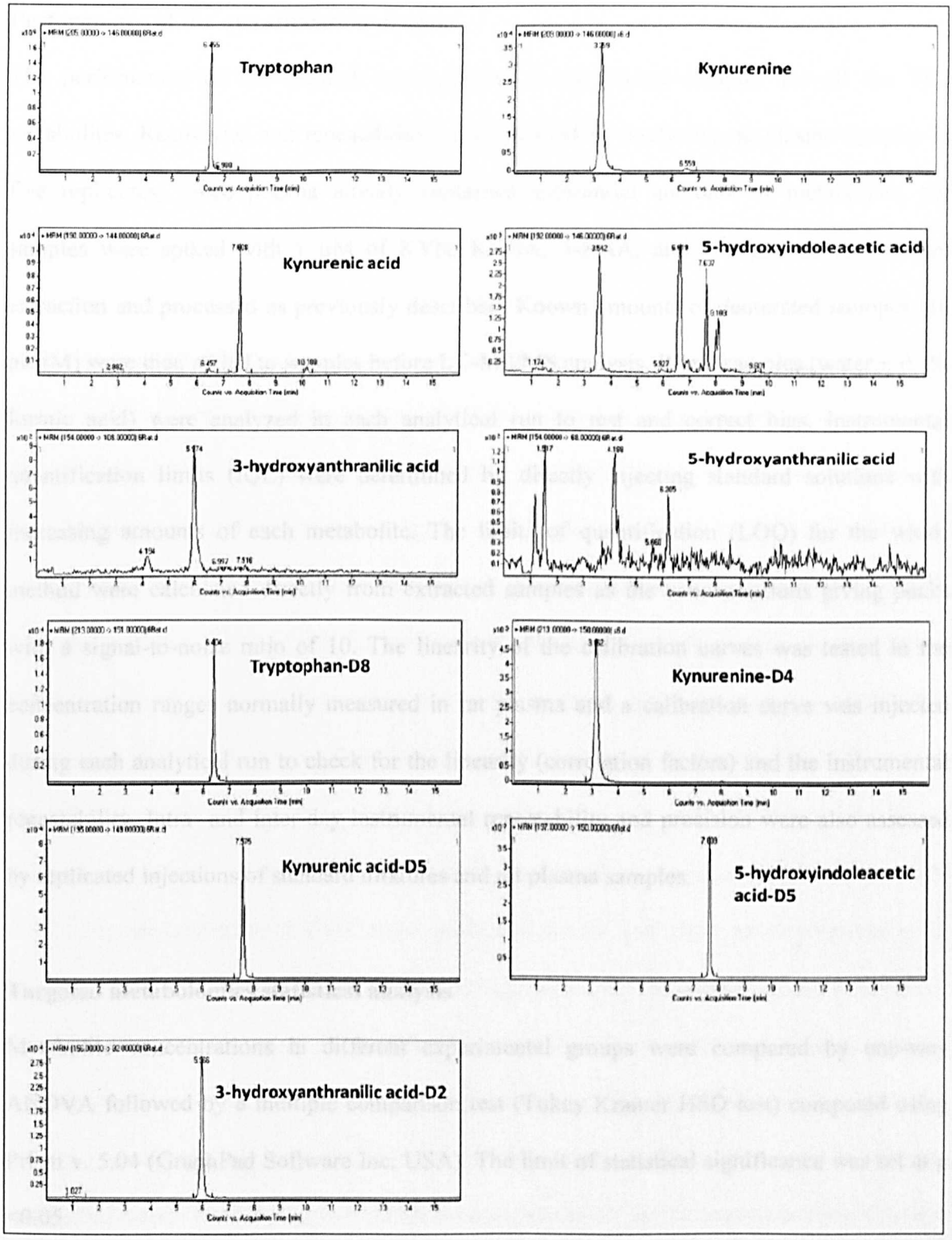


Figure 20. HPLC-MRM chromatograms of tryptophan, L-kynurenine, kynurenic acid, 3-hydroxyanthranilic acid, 5-hydroxyanthranilic acid, 5-hydroxyindoleacetic acid and their deuterated isotopes in rat plasma sample.

Performance of the quantification method

The performance of the method was assessed in rat plasma samples for all the TRP metabolites. Recoveries and repeatability were assessed by analyzing rat plasma samples in five replicates. Since plasma already contained substantial amounts of metabolites, the samples were spiked with 1 µM of KYN, KYNA, 3-HAA, and 180 µM of TRP before extraction and processed as previously described. Known amounts of deuterated isotopes (10 picoM) were then added to samples before LC-MS/MS analysis. Blank samples (water + 0.1% formic acid) were analyzed in each analytical run to test and correct bias. Instrumental quantification limits (IQL) were determined by directly injecting standard solutions with increasing amounts of each metabolite. The limits of quantification (LOQ) for the whole method were calculated directly from extracted samples as the concentrations giving peaks with a signal-to-noise ratio of 10. The linearity of the calibration curves was tested in the concentration ranges normally measured in rat plasma and a calibration curve was injected during each analytical run to check for the linearity (correlation factors) and the instrumental repeatability. Intra- and inter-day instrumental repeatability and precision were also assessed by replicated injections of standard mixtures and rat plasma samples.

Targeted metabolomics statistical analysis

Metabolite concentrations in different experimental groups were compared by one-way ANOVA followed by a multiple comparison test (Tukey Kramer HSD test) computed using Prism v. 5.04 (GraphPad Software Inc, USA). The limit of statistical significance was set at $p < 0.05$.

- **STUDY 2: Validation of KP activation after cardiac arrest with experimental models in rats and pigs and in a small cohort of cardiac arrest patients**

Rats

Animal preparation - Twenty four male Sprague-Dawley rats (Harlan, Italy) weighing 460 ± 17 g were used for the study. Animals were housed two per cage in a temperature (21 ± 1 °C) and humidity (60%) controlled environment. The light schedule was 12 hr light and 12 hr dark, light on at 7:00 a.m. Animals were fasted overnight except for free access to water. The details of the animal preparation were published previously *(Sun 2010)*. In brief, the animals were anesthetized by intra-peritoneal injection of pentobarbital (50 mg/kg), and additional doses (10 mg/kg) were administered at intervals of approximately 1 hr or when required to maintain anesthesia, except that no anesthetic agents were administered for 30 min before induction of cardiac arrest. The trachea was orally intubated with a 14-gauge cannula. A PE-50 catheter (Becton Dickinson, Franklin Lakes, NJ) was advanced into the descending aorta from the left femoral artery for measurement of arterial pressure and sampling arterial blood. Through the left external jugular vein, another PE-50 catheter was advanced into the right atrium for measurement of right atrial pressures. Aortic and right atrial pressures were measured with reference to the mid chest with high-sensitivity transducers. A 3-F PE catheter (model C-PMS-301J, Cook Critical Care, Bloomington, IN) was advanced through the right external jugular vein into the right atrium. A pre-curved guide wire supplied with the catheter was then advanced through the catheter into the right ventricle and confirmed by endocardial electrocardiogram for inducing VF. All of the catheters were flushed intermittently with saline containing 2.5 IU/mL of crystalline bovine heparin. A conventional lead II electrocardiogram was continuously monitored. Temperature was continuously monitored with the aid of a rectal probe and maintained at 37 ± 0.5 °C throughout the experiment.

Experimental procedures - Fifteen min before inducing VF, baseline measurements were obtained and mechanical ventilation was initiated with an inspired FiO2 of 0.21. VF was electrically induced with progressive increases in 60-Hz current to a maximum of 4 mA delivered to the right ventricular endocardium. The current flow was continued for 3 min to prevent spontaneous defibrillation. Mechanical ventilation was stopped after the onset of VF. Precordial compression was begun after 6 min of untreated VF with a pneumatically driven mechanical chest compressor as previously described *(Sun 2010)*. Coincident with the start of precordial compression, animals were mechanically ventilated at a frequency of 50/min with a tidal volume 0.6 ml/100g and a FiO2 of 1.0. Precordial compression was maintained at a rate of 200/min with equal compression-relaxation duration (i.e., 50% duty cycle) and a depth of compression equal to 25% of the animal's anterio-posterior chest diameter. Epinephrine (0.02 mg/kg) was injected into the right atrium 2 min after the start of precordial compression. After 6 min of CPR, resuscitation was attempted with up to three 2 Joule defibrillations (CodeMaster XL, Philips Heartstream, Seattle, WA). Successful resuscitation was defined as the return of supraventricular rhythm with a mean aortic pressure (MAP) > 50 mmHg for a minimum of 5 min. Following resuscitation, animals were monitored for 4 hr. All catheters and endotracheal tubes were then removed. The animals were returned to their cages and were observed for up to 3 days after resuscitation. Animals were sacrificed with an intra-peritoneal injection of pentobarbital sodium (150 mg/kg) at different intervals: baseline, before cardiac arrest (healthy rats, n=7); 2 hr post-resuscitation (n=6); 4 hr post-resuscitation (n=6); and 3 days post-resuscitation (n=5). Plasma was withdrawn for KP assessment and biochemical analyses. Blood was collected into EDTA-tubes and centrifuged for 15 min at 3.000 rpm at 4 °C. Plasma samples were then stored at -80 °C. The heart was quickly removed from the thoracic cavity and frozen at -80 °C for biochemical analyses.

Measurements - Aortic, right atrial pressures, and ECG were continuously recorded on a personal computer-based data acquisition system supported by CODAS hard hardware and software (DataQ, Akron, OH). Coronary perfusion pressure was calculated as the difference between aortic and time-coincident right atrial pressures. Myocardial function was assessed by transthoracic echocardiography (Aloka SSD-5500, Tokyo, Japan) by using a 13-MHz linear transducer at high frame rate imaging (57 Hz). Short- and long-axis 2D views and M-mode were analyzed in real-time and recorded on a magneto-optical disk for off-line analysis. Anterior and posterior end-diastolic and end-systolic wall thicknesses and LV internal dimensions were measured, as recommended by the American Society of Echocardiography, as previously reported *(Fiordaliso 2005)*. LV end-diastolic volume, LV end-systolic volume, and LV EF were calculated by modified Simpson's single-plane rule from a long-axis view. Aortic outflow and transmitral LV inflow velocities were measured from 5 and 4 apical chamber views respectively by pulsed- wave Doppler. All Doppler spectra were recorded for 5-10 cardiac cycles at a sweep speed of 100 mm/s. The color Doppler preset was at a Nyquist limit of 0.44 m/s. Plasma high sensitivity cardiac troponin T (hs-cTnT) concentration was assessed with an electrochemiluminescence assay (ECLIA, Elecsys 2010 analyzer, Roche Diagnostics, Germany). Heart isoprostanes were assessed by a commercially available ELISA (Cayman Chemical, Ann Arbor, MI, USA) method according to the manufacture's instruction.

Pigs

Animal preparation - Ten male domestic pigs, 4 months of age, were used for this study *(Fries 2012)*. The animals were supplied by a single-source breeder, weighed 36 ± 2 kg, and were housed in an air-conditioned room with a 12-hr light-dark cycle from 6:00 am to 6:00 pm. Pigs were anesthetized with an intramuscular injection of 4 mg/kg azaperone, followed by an ear vein injection of 15 mg/kg sodium pentobarbital. The anesthetized animals were then

intubated and mechanically ventilated (Sulla 808-V, Dräger AG, Lübeck, Germany) with a FiO2 of 0.21 and a tidal volume of 15 mL/kg. A continuous infusion of pentobarbital (4 mg/kg/hr) was maintained during the preparation period but was discontinued 30 min before the induction of VF. Thirty min after successful resuscitation, anesthesia was resumed until 2 hr before animals were weaned from the ventilator and brought back to their housing. The respiratory frequency was adjusted to maintain an end-tidal CO_2 between 35 and 40 mmHg. Sonographic imaging of the groin allowed for the percutaneous insertion of catheters into femoral artery and vein. Under fluoroscopy, a fluid-filled catheter was advanced from the left femoral artery into the abdominal aorta (AKS-1830, CODAN pvb Medical, Forstinning, Germany), and a pentalumen pulmonary artery flotation catheter was flow-directed from the left femoral vein into the pulmonary artery (744F75, Edwards Lifesciences, Irvine, CA). To induce VF, a 5F pacing catheter was advanced (again, under fluoroscopy) from the surgically exposed left cephalic vein into the right ventricle. The blood temperature was maintained at 38.2 ± 0.2°C during the preparation phase using a convective blanket that allowed for either heating or cooling the animals (Warm Touch 5200, Tyco Healthcare, Pleasanton, CA). To ensure adequate hydration, a continuous infusion of Ringer's solution was administered at 4 mL/kg/hr throughout the experiment *(Fries 2012)*.

Experimental procedures – Cardiac arrest was induced with 1–2 mA of alternating current delivered to the endocardium of the right ventricle, resulting in VF. Simultaneously, mechanical ventilation was discontinued. Ten min after the onset of VF, precordial compression was initiated using a piston-driven chest compressor (Thumper 1007, MI Instruments, Grand Rapids, MI) at 100 compressions per min. The compressions were synchronized with the simultaneously restarted mechanical ventilation to provide a compression/ventilation ratio of 30:2, with equal compression/relaxation intervals (i.e., a 50%

duty cycle) and a compression depth of 25% of the chest diameter. The ventilation was adjusted to deliver a tidal volume of 15 mL/kg and the FiO2 was increased to 1.0. After 1 min of precordial CC, a bolus dose of 0.03 mg/kg epinephrine was injected into the right atrium via the pulmonary artery flotation catheter. After 6 min of precordial compression, DF was attempted with up to two 150-J biphasic shocks (M-Series CCT, Zoll Medical Corporation, Chelmsford, MA). If an organized rhythm with a MAP of > 60 mmHg persisted for 5 min, the animal was regarded as successfully resuscitated. If VF was not successfully reversed, 1 min of CC preceded the delivery of another sequence of up to two shocks *(Fries 2012)*. After successful resuscitation, animals were randomized to therapeutic hypothermia (n=5) or normothermia (n=5). To mimic a relevant clinical scenario, hypothermia was initiated 1 hr after successful resuscitation and was induced by infusing 1 L of ice-cold (6°C) saline over a period of 30 min. In addition, surface cooling was performed using ice-water-filled bags that were positioned on the inguinal region. A target temperature of 33°C was maintained by the use of the above-mentioned convective blanket. To avoid shivering during the procedure, all animals, including the control group, received a continuous infusion of 0.1 mg/kg/hr pancuronium. At the end of the 16 hr cooling period, the animals were allowed to rewarm slowly at a rate not exceeding 1°C/hr. Animals were ventilated with a FiO2 of 0.3 during the whole post-arrest period. To further evaluate the role of KP pathway in the pathophysiology of post-cardiac arrest syndrome and more specifically of post-cardiac arrest brain injury, KP metabolites were also assayed in an additional group of pigs (n=5), subjected to the same experimental procedure described above, with the exception of an additional known brain protective treatment, namely xenon. Thus, after successful resuscitation, FIO2 was reduced to 30% and the animals were ventilated for 1 hr with a gas mixture of 70% xenon *(Fries 2008)*.

To ensure adequate pain relief, all pigs received an intramuscular injection of 0.1 mg/kg buprenorphine. Catheters were then removed and animals weaned from the ventilator.

Following the extubation, every animal was observed for at least 30 min to ensure adequate spontaneous breathing before being returned to their room. Animals were observed for additional 5 days *(Fries 2012)*.

Measurements - Dynamic data, including MAP, were continuously measured and recorded (AS/3 Compact; Datex-Ohmeda, Achim, Germany). CO and blood temperature were measured and recorded using a Vigilance monitor (Edwards Lifesciences, Irvine, CA). On each day post arrest, animals were evaluated using a validated neurologic deficit score (NDS), as has been described in previous studies *(Fries 2012)*. In brief, the test consisted of four items representing the level of consciousness, respiration, posture, and feeding behavior. Each of the items was graded by severity and given a score. A score of 100 represented no neurologic impairment, while 0 signified brain death. Five days after successful resuscitation, animals were re-anesthetized as described above. After surgical exposure, large-bore catheters were introduced into both carotid arteries and external jugular veins. A buffered 4% paraformaldehyde solution was then anterogradely injected into the carotid arteries until the effluent from the external jugular veins cleared. The brains were then carefully removed from the skulls and placed in identical fixatives for 14 days. Standardized coronal slices were taken at a thickness of 4–5 mm, resulting in a total of 14–15 slices. The anterior and posterior CA1 sectors, respectively, of the hippocampus and occipital neocortex were chosen as the regions of interest, and were then paraffin embedded. In addition to conventional hematoxylin and eosin staining, immunohistochemical reactions were used to visualize reactive astrogliosis (polyclonal rabbit anti-glial fibrillary acidic protein). An experienced neuropathologist, who was blinded to the treatment of the animals, graded the degrees of histopathological alteration *(Fries 2012)*. Briefly, for each region of interest, the proportion of neurons with hypereosinophilia, shrunken cytoplasm, and pyknotic nuclei (which are indicative of

ischemically induced necrotic damage) was graded into five categories (1 = 0%–10%; 2 = 10%–20%; 3 = 20%–50%; 4 = 50%–80%; and 5 = 80%–100%). Reactive astrogliosis was graded on a semiquantitative, 4-point scale (0 = absent; 1 = mild; 2 = moderate; and 3 = severe). The results of these scales for each region of interest were then summed to yield an overall neurohistopathological severity score.

Humans

Patients – Clinical validation of animal results was performed in plasma obtained from 5 patients resuscitated from non-traumatic out-of-hospital cardiac arrest. These patients were prospectively studied in another trial that has been previously published and in which the influence of TH on S-100b values after cardiac arrest has been studied *(Derwall 2009, Stoppe 2012)*. Exclusion criteria were age less than 18 years, severe pre-existing conditions including sepsis, stroke, previous CPR and cancer. Cardiac arrest was defined as the absence of respiration, palpable pulse and responsiveness to stimuli. CPR was performed in accordance to the European Resuscitation Council's (ERC) guidelines 2005. After recovery of blood pressure and pulse for more than 1 hr after admission to the hospital, CPR was considered as "successful" and patients were included in this study *(Derwall 2009, Stoppe 2012)*. After completion of CPR and admission to the hospital, all patients were transferred to the ICU and received standardized intensive care treatment including mechanical ventilation, fluid substitution, tight glucose control, sepsis and vasopressor treatment. The initiation of mild TH was left at the discretion of the attending physicians since not being a standard recommendation in the earlier ERC guidelines. TH was induced using ice bags and infusion of cold fluids. Among the 5 cardiac arrest patients included in the study, 3 received hypothermia treatment, while 2 did not. Tracheal extubation was performed when standard extubation criteria were fulfilled. Patients were discharged from the ICU after fulfillment of standardized

clinical discharge criteria. Three healthy volunteers served as control for plasma measurement of KP metabolites.

Data collection - Clinical data were collected 1 hr after ICU admission and 3 days later, using a web-based data entry system complying with the Utstein-Style, initiated by the German Society of Anaesthesia and Intensive Care Medicine as part of a quality assurance system. A standardized neurological assessment was performed by an independent physician, using the CPC after 14 days. CPC 1 and 2 were considered as favorable neurological outcome, whereas CPC 3 to 5 labeled adverse outcome *(Derwall 2009, Stoppe 2012).* Serum samples for the determination of C-reactive protein (CRP), PCT, tumor necrosis factor alpha (TNF-a), IL-6 and 8, macrophage inhibitor factor (MIF), and KP metabolites, were taken at the same time points. The inflammatory cytokines IL-6, IL-8, TNF-a and the biomarkers CRP, PCT were quantified using commercially available automated systems (LIAison, DiaSorin, Dietzenbach, Germany, and KRYPTOR, Brahms AG, Hennigsdorf, Berlin, Germany). The serum levels of MIF were determined using an enzyme-linked immunosorbent assay (ELISA) *(Stoppe 2012).*

TRP and KP metabolites measurements

Absolute quantification of plasma TRP and KP metabolites was performed by LC-MRM coupled with isotope-dilution MS. Metabolites assayed included: TRP; KYN; KYNA; 3-HAA. Plasma samples (20 µL) from rats, pigs and humans were spiked with 10 µM of deuterated standards (tryptophan-D8, L-kynurenine-D4, kynurenic acid-D5, 3-hydroxyanthranilic acid-D2). Spiked plasma samples were then deproteinized by mixing with four volume of cold methanol, vortexed, and incubated at -20°C for 1h. Samples were centrifuged 10 min at 14.000 x g, the supernatant was collected, and the centrifugation was repeated. The supernatant was dried in a SpeedVac and re-suspended in 20 µL of 0.1% formic acid. Ten µL of supernatant

were analyzed directly by LC- tandem MS, with the Agilent 1200 series system for LC. Separation was performed with a Synergy 4u Fusion-RP 80A column (50x2.00 mm, Phenomenex) using as mobile phase A 0.1% formic acid in water and mobile phase B 100% acetonitrile at a flow rate of 0.2 mL/min. Elution started with 99% of A and 1% of B, followed by a 13-min linear gradient to 99% of B, a 2-min isocratic elution and a 1-min linear gradient to 99% of A, which was maintained for 8 min to equilibrate the column. The mass spectrometric analysis was performing using an Agilent 6410 triple quadrupole mass spectrometer (Agilent Technologies) in positive ion mode for all the metabolites. Quantitative analyses were processed with MassHunter workstation quantitative analysis software v B.01.04 (Agilent Technologies). Assay performance in terms of linearity, sensitivity, analytical recovery and instrumental repeatability was assessed in plasma samples for all the tryptophan metabolites. The instrumental sensitivity was good and the IQLs ranged from 0.0015 to 0.25 pmoles/injected. LOQs in rat plasma ranged between 0.55 and 6 nM. The recoveries in plasma were higher than 60% for all the metabolites. The analytical response was linear for all the compounds in the range of concentrations measured in plasma and the inter-day correlation factors (r2) were $\geq$ 0.9994 with standard deviations (SD) $\leq$ 0.0007. Instrumental repeatability, assessed using replicate injections of standard mixtures and plasma, was generally $\leq$ 10%.

Statistical analysis

Normal distribution of the data was confirmed using the one sample Kolmogorov-Smirnov Z test. For comparisons among time-based measurements within groups, one-way ANOVA with Tukey Kramer's multiple comparisons was used. Linear correlations were calculated using the Pearson correlation coefficient. All data are reported as mean ± SEM. A 2-tail $p < 0.05$ was

considered as statistically significant. All analyses were performed by SPSS 16 (SPSS Inc, Chicago, IL).

- **STUDY 3: Validation of KP activation after cardiac arrest in a large cohort of out of hospital cardiac arrest patients**

Included patients - Patients included in the present study were part of the FINNRESUSCI study, which was a prospective observational cohort study conducted at 21 hospitals in Finland between March 1st 2009 and February 29th 2010 *(Vaahersalo 2013)*. The study was approved by the ethic committees of each participating hospital. Informed consent from the patient next of kin was obtained for data collection and blood sampling. For this study we included patients with VF/VT as the initial rhythm, in whom blood samples were obtained at ICU admission. Blood was drawn also from 10 healthy volunteers who were matched by age and sex with patients.

Blood samples - Blood samples were collected into ethylenediaminetetraacetic acid (EDTA) tubes, centrifuged and plasma stored at -70°C. Upon analysis, samples were thawed and divided into aliquots. Plasma levels of TRP and its metabolites, KYN, KYNA and 3-HAA were measured blinded to case identity using HPLC coupled MS. We used blood samples from healthy subjects to serve as controls. Briefly, human plasma samples (100 µL) were mixed with 10 µL of internal standard (IS, 5-HTRP, 1 ng/µL final concentration), deproteinized by adding 400 µL of cold methanol and incubating for 1h at -20°C. After sample centrifugation for 10 min at 14.000x*g*, the supernatants were collected and centrifuged again. Supernatants were dried under nitrogen flow, and residues were dissolved in 100 µL of

1% acetonitrile in 0.1% formic acid, transferred to autosampler vial insert and 40 µL of supernatant were injected directly in tha HPLC system (Alliance separation module 2695, Waters, Milford, MA, USA). The chromatographic separation was obtained with an Accucore PFP column (150 × 2.1 mm; 2.6 µm particle size Thermo-Scientific), at a flow rate of 0.2 mL/min. Elution started with 99% of mobile phase A (0.1% formic acid in water) and 1% mobile phase B (100% acetonitrile) for 2 min, followed by a 18-min linear gradient to 50% of A, a 1-min linear gradient to 30% of A and a 1-min linear gradient to 99% of A which was maintained for 12-min to equilibrate the column. The total run time was 35 min. The mass spectrometric analysis was performing using a Micromass Quattro Micro API triple-quadrupole (Waters, Milford, MA, USA) in positive ion mode and MRM mode, measuring the fragmentation products of the deprotonated pseudo-molecular ions. The choice of fragmentation products for all compounds and the optimization of collision-induced dissociation energies (EC) were done in continuous-flow mode, using standard solutions at concentrations of 10 ng/µL for all compounds. Data were processed with the MassLynx software (Waters, Milford, MA, USA). Plasma concentrations of TRP and KYN were expressed in micromolar, and KYNA and 3-HAA in nanomolar.

Data collection - The participating hospitals were a part of the Finnish Intensive Care Consortium (FICC) and all 21 ICUs used the same electronic data management systems and data validation software (Web Validator, Tieto, Helsinki, Finland). Data of study patients were prospectively collected using an Internet-based case report forms (CRF). Pre-hospital data was collected by the paramedics in accordance with the Utstein Guidelines and included whether the arrest was witnessed or not, the administration of bystander initiated life support (BLS), time from call to the dispatch centre and to ROSC and the use of adrenaline *(Langhelle 2005)*. In-hospital care data were collected electronically and comprised the Simplified Acute

Physiology Score (SAPS) II score and ICU and hospital mortality. For the present trial we used SAPS II score derived systolic blood pressure (SAP) and bicarbonate to define the severity of post cardiac arrest shock.

Survival and neurological outcome - Time of death was recorded for each patient. A specialist in neurology blinded to management in the ICU contacted patients discharged from the hospital by phone one year after cardiac arrest and determined neurological outcome according to the CPC score *(Langhelle 2005)*. We defined good neurological outcome as CPC (1-2) and poor neurological outcome as CPC (3-5).

Statistical analysis - Descriptive statistics were calculated using counts and percentages for categorical variables. Median values with interquartile range (IQR) were calculated for continuous variables. Levels of KP metabolites across variables at admission were compared by Chi-square, Mann-Whitney U and the Kruskal-Wallis test and changes over time were analysed using the Wilcoxon signed rank test for paired data. Multivariable linear regression was used to identify the independent factors at resuscitation influencing metabolite levels on ICU admission. Results of linear regression are reported in terms of beta coefficients with 95% confidence interval (CI) and p-values. Multivariable logistic regression was used to identify factors at resuscitation that were predictors of ICU mortality and poor neurological outcome at 12 months from the arrest. All variables associated with the outcome in univariate analysis ($p<0.05$) were included in the multivariable model. Each KP metabolite was included separately in the model as continuous variables. Odds ratios (OR) with the corresponding 95% CI were calculated and p values were considered statistically significant if they were less than 0.05. The addition of new predictive variables into a model was assessed by comparing the area under the curve (AUC) between models using a non-parametric method *(DeLong 1988)*. All

statistical analyses were performed using IBM SPSS version 19.0 and Analyze-it® (Analyse-it Software, Ltd. http://www.analyse-it.com/; 2009)

- ***ECG-DERIVED BIOMARKERS:***

- **STUDY 4: AMSA evaluation in 609 VF patients in the United States**

Included ECGs - A database of ECG traces recorded during pre-hospital CPR, including 1410 DFs, obtained from 748 patients between 2005-2007, was available through the courtesy of ZOLL Medical Corporation (Chelmsford, MA, USA). ECGs were recorded from defibrillation pads using ZOLL AED PLUS and ZOLL AED PRO in multiple emergency medical systems in the United States through a regular field case submission program. The electronic data did not contain any patient identifiable information, accordingly to Health Insurance Portability and Accountability Act (HIPAA) regulations.

ECG analyses - ECGs were recorded at a sample rate of 250 Hz. The AEDs provided a single rectilinear biphasic waveform shock of 120 Joules for the first DF, and 150 or 200 Joules for the subsequent DFs. The AMSA analysis has been previously described *(Young 2004, Ristagno 2008b)*. Briefly, ECG signals were processed using a 2 Hz high-pass filter to minimize low frequency artifacts produced by CC and a 48 Hz low-pass filter to remove interference of ambient noise at higher frequencies. Analog ECG signals were digitized and converted from a time to a frequency domain by FFT. AMSA was calculated as the sum of the products of individual frequencies and their amplitudes (**Figure 21**):

$AMSA = \sum Ai \cdot Fi$

where Ai represented the amplitude at i^{th} frequency Fi.

The analysis was performed during hands off time on a 512 point window (2.05 sec) ending 0.5 sec prior to the DF.

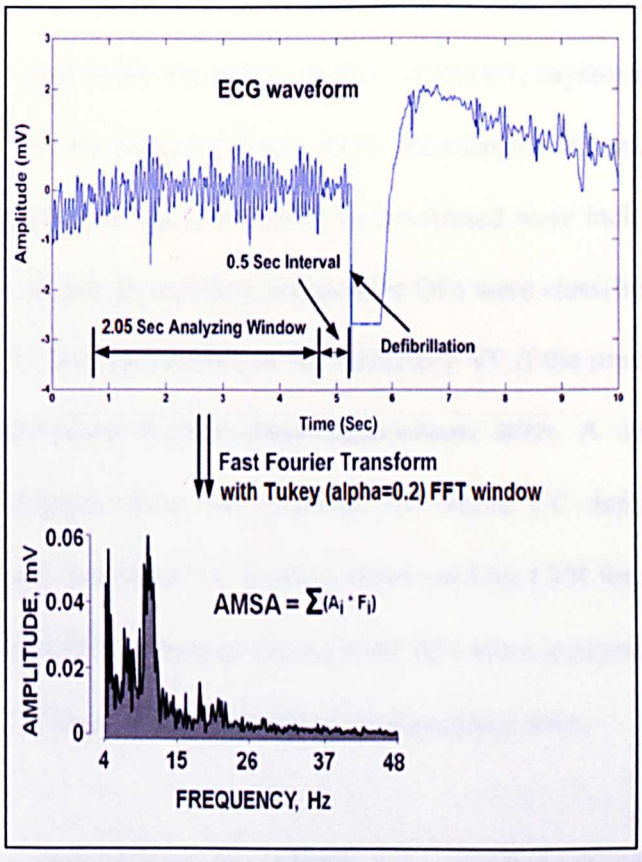


Figure 21. Fast Fourier Transformation with AMSA calculation.

A Tukey FFT window was used to reduce edge effects. More specifically, Tukey window (r=0.2) was calculated as:

$$\mathbf{X(K)} = \sum_{j=1}^{N} \mathbf{x(j)}\omega_N^{(j-1)(k-1)}$$

where x(j) is the ECG data points, $\omega_N = e^{\frac{-2\pi i}{N}}$ is an *N*th root of unity and X(K) is the FFT coefficient of the associated frequency component. The absolute value of X(K) was then obtained to represent the amplitude of the signal at the frequency domain.

$\mathbf{A(f) = abs(X(K))}$, f=0:0.488:125, K=1:1:257

For the purpose of this study, the DF outcome was defined according to the following established criteria *(Ristagno 2008b)*: "successful defibrillation" or return of a potentially perfusing rhythm, if DF restored an organized rhythm with heart rate ≥ 40 beats/min commencing within 60 sec post shock; and "unsuccessful defibrillation" or failure of return of a potentially perfusing rhythm, if VF/VT, asystole, or pulseless electrical activity with pauses > 5 sec occurred. Only ECG recordings with adequate pre- and post- DF durations and in which DF outcome could be confirmed were included in the study: 1260 instances from 609 patients. In addition, subsequent DFs were classified as being for recurrent VF if the preceding DF was successful, or for refractory VF if the preceding DF had failed to restore a potentially perfusing rhythm *(Shanmugasundaram 2012)*. A sub-group analysis was conducted on DFs obtained from 303 patients, for whom CC depth data were available. Depth of CC was measured from an accelerometer used for CPR feedback and registered by the AEDs. Changes in AMSA between consecutive DFs were analyzed in relationship to the 2005 recommended CC depth > 1.5 inches (in) *(AHA guidelines 2005)*.

Statistical analysis - AMSA was computed using Matlab 7.2 (MathWorks, Natick, MA). Two independent readers reviewed the ECG recordings to confirm DF outcomes. Differences in AMSA between DFs were analyzed by analysis of variance (ANOVA) with Scheffe's method for multiple comparisons. A range of AMSA thresholds was evaluated. The sensitivity, defined as the capability of AMSA to identify DFs that successfully reestablished a potentially perfusing rhythm, was calculated as the number of correctly predicted successful DFs divided by the total number of successful DFs. The specificity, which refers to the capability of AMSA to identify failure of a DF, was calculated as the number of correctly predicted unsuccessful DFs divided by total number of unsuccessful DFs. The PPV referred to the proportion of DFs that were correctly predicted by AMSA to restore a potentially perfusing

rhythm. The NPV represented the proportion of DFs that were correctly predicted by AMSA to fail. The accuracy was calculated as the proportion of true results (both true positively predicted successful DFs and true negatively predicted unsuccessful DFs) in the population. Finally, ROC curve analysis was performed. SPSS 16.0 (SPSS Inc., Chicago, IL) was used. A value of $p<0.05$ was regarded as statistically significant. Data are presented as mean ± SEM.

- **STUDY 5: AMSA evaluation in 1.617 VF patients in Lombardia region, Italy**

Included ECGs and patients - A database of ECG traces recorded during pre-hospital CPR, including 2.442 DFs, obtained from 1.050 patients enrolled in 9 cities in Regione Lombardia, Italy between 2008-2009, was used as derivation group. An additional database of ECG traces recorded during pre-hospital CPR, including 1.386 DFs, obtained from 567 patients enrolled in the same cities in Regione Lombardia, Italy in 2010, was used as validation group. ECG traces were available through the courtesy of the EMS Regional Coordinating center "Azienda Regionale Emergenze Urgenze" (AREU), Milan, Italy. The electronic data did not contain any patient identifiable information. The study was approved by the ethic committee of coordinating hospital, San Gerardo University hospital, Monza, Italy.

Data collection - The participating EMSs from the different cities were part of the AREU and all used the same electronic data management system and data validation software (EMMAweb, AREU, Milan, Italy). Data were prospectively collected using an Internet-based CRF. Pre-hospital data were collected by the paramedics in accordance with the Utstein Guidelines. In-hospital data, collected and stored electronically at the Regional database, comprised: co-morbidities and drug treatment (over the last year prior to cardiac arrest),

hospital survival, 6 month and 1 year survival. For the derivation group, data from the Regional database were retrieved and linked to the EMMAweb database by the Department of Clinical Medicine and Prevention (DIMEP), University of Milano-Bicocca, Italy.

ECG analyses - ECG parameters were computed using Matlab 7.2 (MathWorks, Natick, MA). ECGs were recorded from defibrillation pads using different AEDs: A. ZOLL Medical Corp. (biphasic rectilinear DF waveform); B. Philips Health System (biphasic truncated DF waveform); and C. PhysioControl Inc (biphasic truncated DF waveform), as reported in **Figure 22**. The AEDs provided a single shock every 2 min of CC. ECGs were recorded at different sample rates: 250 Hz for AED A; 200 Hz for AED B; and 125 Hz for AED C.

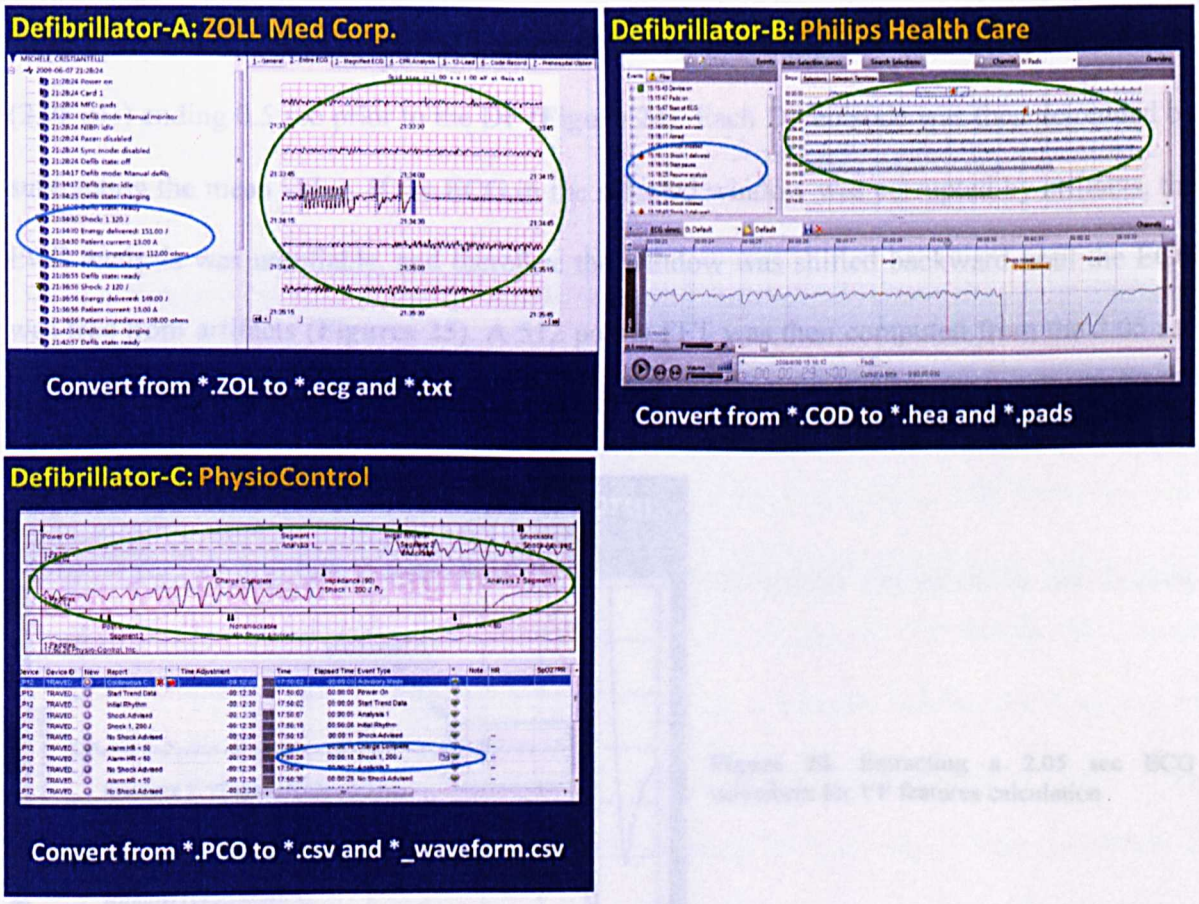


Figure 22. ECG traces and shock information recorded from the three different AEDs.

For each type of AED, the recorded waveforms were exported to a Matlab format for further processing. The ECG traces were then re-sampled at 250 Hz using a polyphase filter implementation. The re-sampling process was necessary due to different sample rates employed by the different AEDs (**Figure 23**).

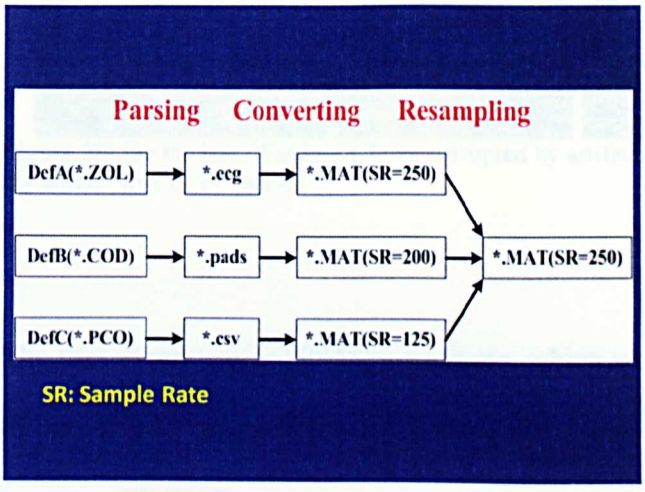


Figure 23. Converting ECG trace files into a uniform .MAT format at a unique sample rate of 250 Hz.

After re-sampling, the analysis was performed during hands off time on a 512 point window (2.05 sec) ending 0.5 sec prior to the DF (**Figure 24**). Each DF episode was then detrended by subtracting the mean value. If the ECG in the selected window was corrupted by artifacts, the ECG analysis was unreliable, and therefore the window was shifted backward until the ECG was free from artifacts (**Figures 25**). A 512 points FFT was then computed from the 2.05 sec data with a Tukey window, as reported in STUDY 4.

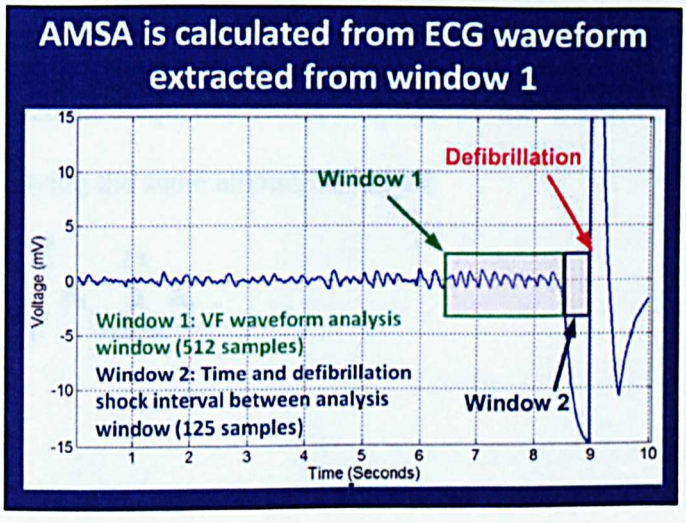


Figure 24. Extracting a 2.05 sec ECG waveform for VF features calculation

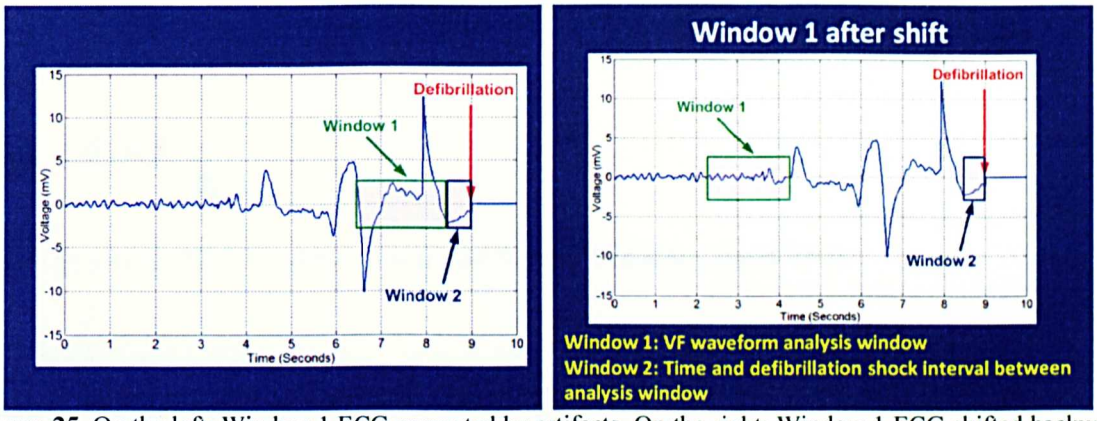


Figure 25. On the left: Window 1 ECG corrupted by artifacts. On the right: Window 1 ECG shifted backward to the artifact free ECG interval.

The root mean square (RMS) amplitude, which was defined as the square root of the average of the squared values of the data points, was calculated as:

$$A_{RMS} = \sqrt{\frac{1}{N}\sum_{i=1}^{N} x_i^2}$$

where x(i) denotes sample i in a detrended ECG episode of length N.

Peak frequency (PF), which denotes the frequency that corresponding to the maximum power, was given by:

$$PF = \arg\max[\widehat{A}(f)]$$

Median frequency (MDF) represents the frequency that divides the signal in two regions having the same amount of power:

$$\sum_{f1}^{MDF} A_i \sum_{=MDF}^{f1} A_i$$

Mean frequency (MNF), which provides a smooth estimate of the concentration of spectral power, is computed as:

$$MNF = \sum_{f1}^{f2} A_i F_i \Big/ \sum_{f1}^{f2} A_i$$

where f1 and f2 (f2>f1) are the lower and higher frequency band limits.

AMSA, was calculated as reported in the STUDY 4.

DF outcome was defined according to the criteria reported above in STUDY 4 *(Ristagno 2008 and 2013b)*: "successful defibrillation" or return of a potentially perfusing rhythm, if DF restored an organized rhythm with heart rate ≥ 40 beats/min commencing within 60 sec post shock; and "unsuccessful defibrillation" or failure of return of a potentially perfusing rhythm, if VF/VT, asystole, or pulseless electrical activity with pauses > 5 sec occurred. Only ECG recordings with adequate pre- and post- DF durations and in which DF outcome could be confirmed were included in the study (1.050 in the derivation group and 567 in the validation one). In addition, for 860 patients in the derivation group, in-hospital and long-term outcome were known and additional analyses were performed.

Statistical analysis - Descriptive statistics were reported using counts and percentages for categorical variables, mean with SD or median with IQR for continuous variables and counting processes. Normal distribution of ECG-derived parameters was investigated. Linear regression was used to investigate the association between relevant factors and ECG-derived parameters values prior to DF. In the regression models, the natural logarithm of the ECG-derived parameters was the dependent variable and age and gender were always introduced as independent factors. All factors associated with the ECG-derived parameters in the univariate

analysis ($p<0.05$) were included in the multivariate model. Results of linear regression were reported including beta values and p-values. Comparisons of ECG-derived parameters mean values between outcomes were performed using t-test or ANOVA, when adjusted for confounding factors on the natural logarithm of the parameters. Logistic regression was used to investigate the association between ECG-derived parameters and outcomes, i.e. DF outcome, ROSC, and survival at hospital discharge, while Cox regression models were used for survival at 6 months and 1 year. ECG-derived parameters were included separately in the model as continuous variables. OR and Hazard Ratios (HR) with the corresponding 95% CI were reported for logistic models and Cox regression models respectively. We estimated both unadjusted and adjusted relative risk (OR and HR). The estimates were adjusted for age and gender and for factors that were predictors of outcomes in the multivariate models. The discriminatory ability of ECG-derived parameters was measured as AUC using DF success and ROSC as outcomes. AUC with 95% CI and p-values for difference from chance were reported. Differences among AUCs of ECG-derived parameters were tested assuming the mathematical equivalence of the AUC to the Mann-Whitney U-statistic. Finally, sensitivity, specificity, PPV, NPV and accuracy curves were computed to evaluate threshold values of AMSA. All statistical analysis was performed using SAS version 9.2.

RESULTS

- ***CIRCULATING BIOMARKERS:***

- **STUDY 1: Discovery of new circulating biomarkers with the aid of untargeted metabolomics in a rat model of cardiac arrest**

To initially explore the metabolome changes associated with the early post-resuscitation phase, a plasma LC-MS/MS approach was used to discover unbiased small-molecule metabolic profiles in rats not subjected to cardiac arrest (control, CTR) and in rats subjected to cardiac arrest and CPR and sacrificed 2 hr after resuscitation. Mass-spectral data were subject to peak alignment and data pre-processing by SIEVE 1.3. (**Figure 26**).

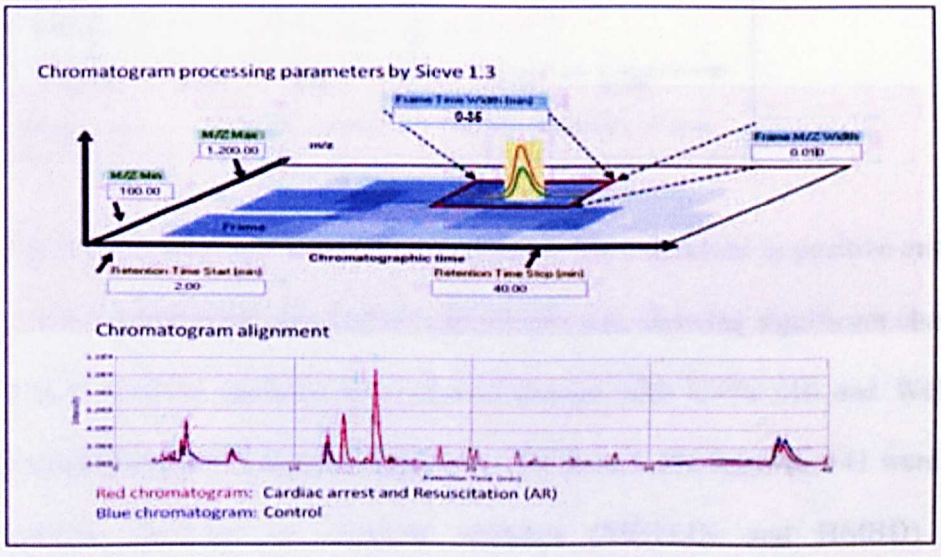


Figure 26. Mass-spectral data were subject to peak alignment and data pre-proccesing by SIEVE 1.3.

Data were then analyzed for global changes by using multivariate statistics to determine group separation as well as univariate statistics to evaluate the number and percentage of features that varied significantly between the 2 sample sets. As seen in **Figure 27**, PCA (principal component 1 vs. principal component 2) revealed an excellent separation of the two experimental groups under both positive and negative modes.

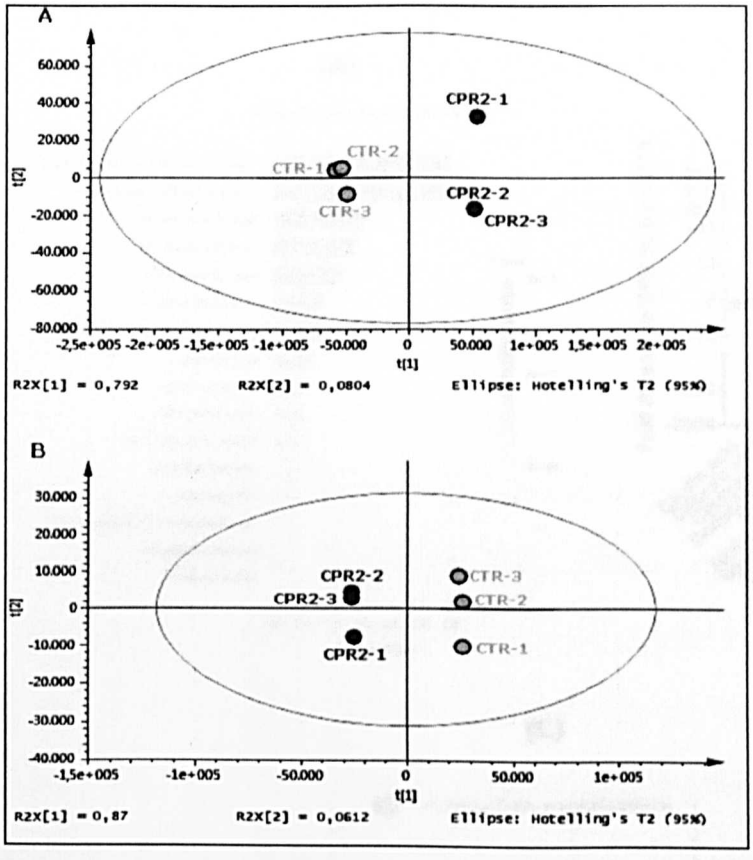


Figure 27. PCA score plots of the plasma profiling of rats not subjected to cardiac arrest as control (CTR) and rats subjected to cardiac arrest and cardiopulmonary resuscitation, euthanized 2 hr after resuscitation (CPR2h). Panel A, with the positive ESI dataset. Panel B, with the negative ESI dataset.

Up to 4.534 and 4.710 features respectively were detected in positive and negative ion mode. 1.306 features were detected in both sample sets, showing significant changes in their relative signal intensity (defined as a ≥2-fold change with CV% ≤10 and Welch's t-test p ≤0.01) (**Supplementary Table S2,** Appendix). Of these 1.306 features, 141 were related to molecular species identified by database searches (METLIN and HMBD) and are listed in **Supplementary Table S3** (Appendix). It should be noted that a given molecule may be represented by several different features, such as naturally occurring components of its isotopic cluster or non-specific adduct ions. Several analytes were detected only in positive mode, while others were observed only in the negative ion mode as already reported for plasma samples. To further interpret the biological significance in the early post-resuscitation phase, we used MetaboAnalyst tools to link these metabolites to metabolic pathways, as described in **Figure 28.**

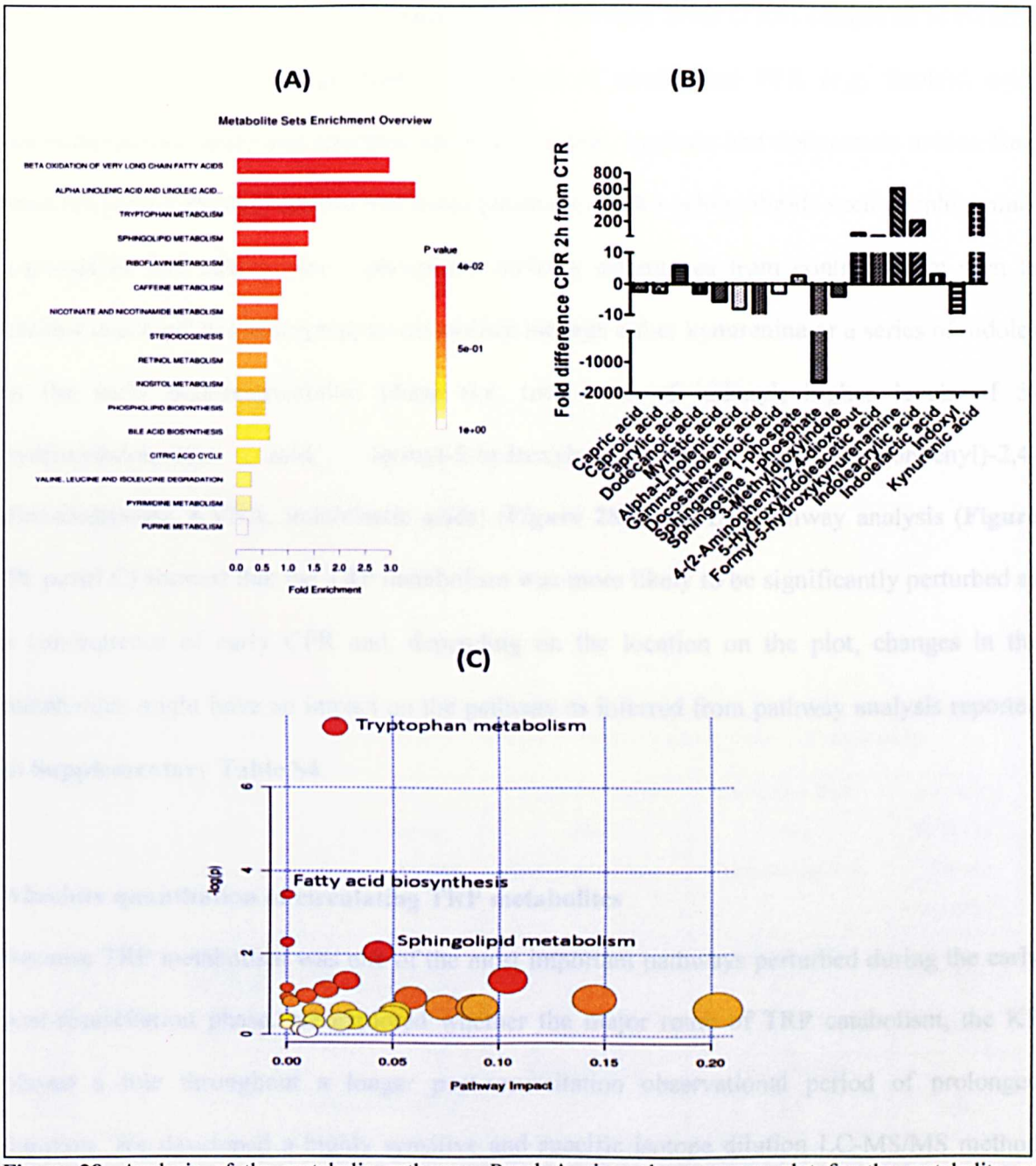


Figure 28. Analysis of the metabolic pathways. Panel A gives the summary plot for the metabolite set enrichment, panel B shows the difference in abundance of the metabolites, mapped into enrichment categories, and panel C shows all metabolic pathways arranged according to the scores from enrichment analysis (y axis) and topology analysis (x axis).

Analysis of the differences between the two datasets indicated that the beta-oxidation of fatty acids, linolenic acid metabolism, tryptophan metabolisms and sphingolipid metabolism were over-represented (**Figure 28**, panel A). The very early CPR phase (2h) showed plasma

changes, although modest, in the composition of free fatty acids (FFA) compared to control. For example there was a generally lower level of unsaturated FFA (*e.g.* linoleic, acid, docosahexaenoic acid) and saturated acids (*e.g.* capric, myristic and dodecanoic acids). Rats from the early CPR interval also had lower plasma levels for sphingolipids such as sphiganine-1-phosphate and sphingosine 1-phosphate. Striking differences from controls were seen in various intermediates of tryptophan catabolism through either kynurenine or a series of indoles in the early post-resuscitation phase (i.e. low level of indoxyl; higher levels of 5-hydroxyindolacetic acid, formyl-5-hydroxykynurenamine, 4-(2-aminophenyl)-2,4-dioxobutanoate, KYNA, indolelactic acids) (**Figure 28**, panel B). Pathway analysis (**Figure 28**, panel C) showed that the TRP metabolism was more likely to be significantly perturbed as a consequence of early CPR and, depending on the location on the plot, changes in the metabolites might have an impact on the pathway as inferred from pathway analysis reported in **Supplementary Table S4.**

Absolute quantitation of circulating TRP metabolites

Because TRP metabolism was one of the most important pathways perturbed during the early post-resuscitation phase, we explored whether the major route of TRP catabolism, the KP played a role throughout a longer post-resuscitation observational period of prolonged duration. We developed a highly sensitive and specific isotope dilution LC-MS/MS method for accurately quantifying TRP, KYN, KYNA, and 3-HAA, in rat plasma at 2 hr after resuscitation, compared to controls. Deuterated metabolites were added to plasma samples as internal standards. We assessed the assay performance in terms of linearity, sensitivity and analytical recovery. The instrumental sensitivity was good and the IQLs ranged from 0.0015 to 0.25 pmoles/injected. LOQs in rat plasma ranged between 0.55 and 6 nM (**Table 7**). The recoveries in rat plasma were higher than 60% for all the metabolites (**Table 7**). The analytical

response was linear for all the compounds in the range of concentrations measured in rat plasma and the inter-day correlation factors (r^2) were ≥ 0.9994 with standard deviations (SD) ≤ 0.0007 (**Table 8**). Instrumental repeatability, assessed using replicate injections of standard mixtures and rat plasma, was generally ≤ 10% (**Table 8**).

Table 7. Tryptophan and its main metabolites (mean recovery ± SD), instrumental quantification limits (IQL), and limits of quantification (LOQ) of the analytical method in plasma (5 replicates).

Chemicals	Recovery % (mean ± SD)	IQL (pmoles/injected)	LOQ (nM)
TRP	89±3.49	0.07	1
KYN	93±2.27	0.01	0.31
KYNA	93±2.17	0.0015	0.55
3-HAA	65±2.36	0.02	5.43

Table 8. Linearity ranges and between-day correlation factors of the calibration curves (r^2 ± SD), intra- and inter-day variability (relative standard deviation-RSD) in analytical standards and in plasma (5 replicates).

Chemicals	Linearity range (microM)	Interday correlation factors (r^2±SD)	Intraday RSD (%) *Standard mixture*	Interday RSD (%) *Standard mixture*	Intraday RSD (%) *Plasma samples*
			1 pmoles/inj	**1 pmoles/inj**	
KYNA	0.025-16	0.9997±0.0002	2.17	1.21	1.67
3-HAA	0.025-16	0.9998±0.0001	7.83	7.13	2.76
			4 pmoles/inj	**4 pmoles/inj**	
KYN	4-64	0.999248±0.0005	6.47	10	5.30
			0.5 nmoles/inj	**0.5 nmoles/inj**	
TRP	12.5-500	0.9989±0.0007	4.49	4.17	9.04

Significant changes in plasma concentrations of TRP and the metabolites showed significant differences from controls 2 hr after resuscitation (**Figure 29**). More specifically, TRP decreased significantly, while KYN, KYNA and 3-HAA increased significantly compared to control animals (**Figure 29**).

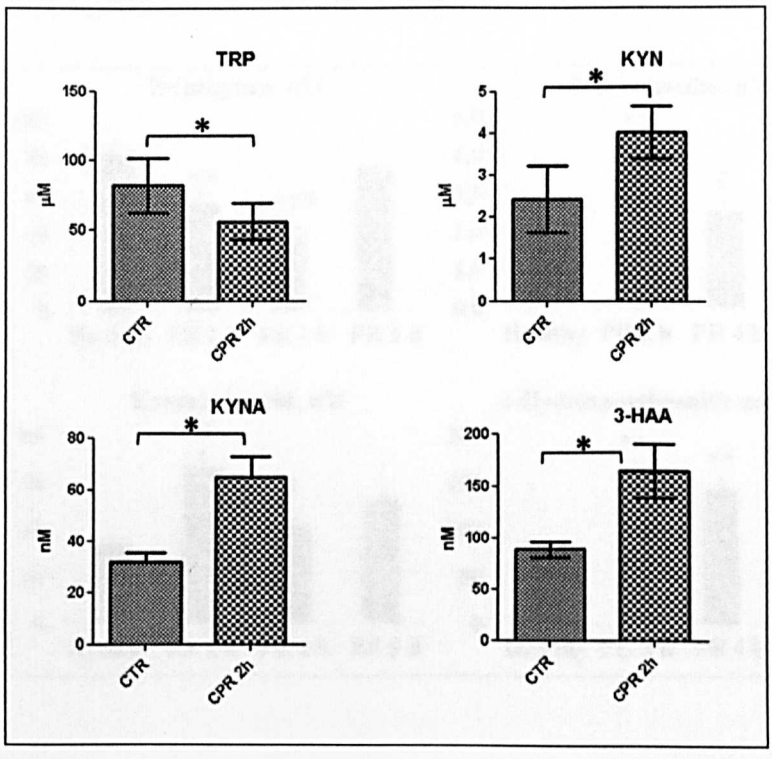


Figure 29. Rat plasma concentrations of tryptophan (TRP) and its metabolites at different cardiopulmonary resuscitation phases (CPR). Each bar represents the metabolites' plasma concentration as mean ± SD (n = 6 rats/group). Asterisks mark significant differences in expression (one-way ANOVA, Tukey–Kramer HSD, $p<0.05$).
CTR, rats not subjected to cardiac arrest, as control; CPR2h, rats subjected to cardiac arrest and CPR, euthanized at 2 hr after resuscitation; KYN L-kynurenine; KYNA kynurenic acid; 3-HAA 3-hydroxyanthranilic acid.

- **STUDY 2: Validation of KP activation after cardiac arrest with experimental models in rats and pigs and in a small cohort of cardiac arrest patients**

Rats. At 2 and 4 hr post-resuscitation resuscitation, plasma levels of TRP were significantly lower in cardiac arrest animals compared to healthy ones ($p < 0.01$, **Figure 30**). Plasma levels of the TRP's metabolite KYN and its derivatives, KYNA and 3-HAA, significantly increased following resuscitation ($p < 0.01$ vs. healthy rats, **Figure 30**). Plasma levels of TRP tended to return to normal values 3 days after resuscitation (**Figure 30**). However, higher plasma concentrations of KYN, KYNA, and 3-HAA persisted ($p < 0.01$ vs. healthy rats, **Figure 30**). Post-resuscitation LV systolic and diastolic dysfunction occurred in each rat, as evidenced by

hemodynamic and echocardiographic data reported in **Table 9**.

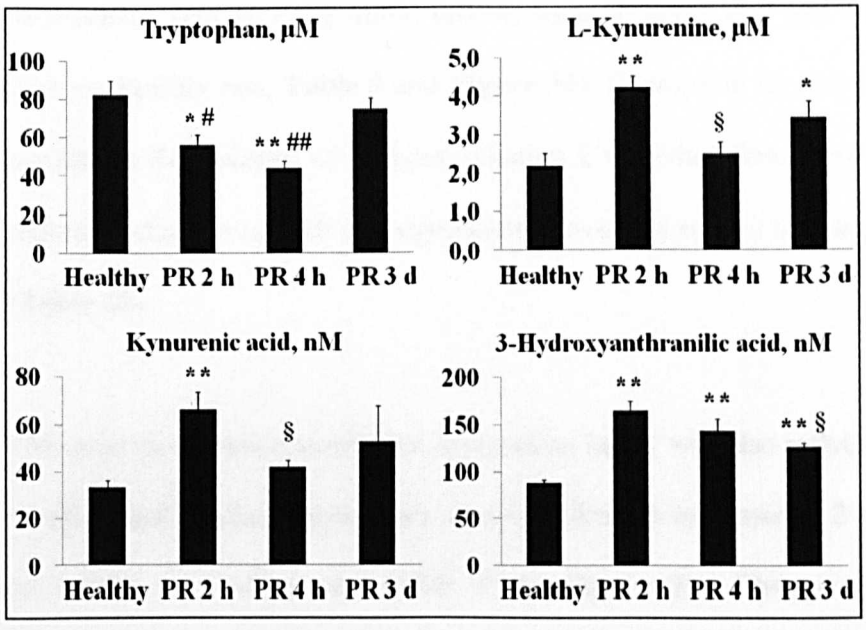


Figure 30. Kynurenine pathway activation in rats resuscitated from cardiac arrest. Plasma concentrations of trypthophan, L-kynurenine, kynurenic acid and 3-hydroxyanthranilic acid in healthy rats and in rats after 2 hours (h), 4 h, and 3 days (d) post-resuscitation (PR).
Data are reported as mean ± SEM (n = 6 rats/group).
* $p < 0.05$ and ** $p < 0.01$ vs. healthy;
# $p < 0.05$ and ## $p < 0.01$ vs. PR 3 days;
§ $p < 0.05$ vs. PR 2 h.

Table 9. Hemodynamics, functional and histological outcome, and biomarkers in resuscitated rats.

	Healthy	PR 2 hr	PR 4 hr	PR 3 days
MAP, mmHg	136 ± 2	105 ± 2**	105 ± 5**	N.A.
CPP, mmHg	116 ± 3	87 ± 2**	91 ± 4**	N.A.
EF, %	79 ± 2	42 ± 5**	48 ± 6**	75 ± 3##§§
EDV, µL	378 ± 24	446 ± 51	465 ± 57	280 ± 22*#§
SV, mL	0.34 ± 0.02	0.21 ± 0.02**	0.18 ± 0.02**	0.22 ± 0.02**
DT, msec	28.0 ± 3.9	18.8 ± 1.2	19.4 ± 1.3	27.6 ± 2.8#§
hs-cTnT, ng/L	57 ± 18	4453 ± 615**	3874 ± 935**	669 ± 531##§
c-Isoprostanes, pg/µg prot	7 ± 2.2	57 ± 8**	47 ± 5**	14 ± 3.2##§§

MAP, mean arterial pressure; CPP, coronary perfusion pressure; EF, ejection fraction; SV, stroke volume; DT, deceleration time; EDV, end diastolic volume; hs-cTnT, high sensitive cardiac troponin T; c-Isoprostanes, cardiac isoprostane; PR, post-resuscitation, (n = 6 rats/group).
** $p < 0.01$ and * $p < 0.05$ vs. Healthy or Baseline; ## $p < 0.01$ and # $p < 0.05$ vs. PR 2 hrs;
§§ $p < 0.01$ and § $p < 0.05$ vs. PR 4 hrs.

More specifically, marked decreases in MAP and CPP, LV EF, stroke volume, and deceleration time of early mitral inflow, were observed at 2 and 4 hr post-resuscitation ($p < 0.01$ vs. healthy rats, **Table 9** and **Figure 31**). Changes in the echocardiographic parameters, evaluating the severity of post-resuscitation LV dysfunction, were significantly and directly related to changes in TRP and significantly inversely related to changes in KYNA and 3-HAA (**Table 10**).

The severity of post-resuscitation myocardial injury was also reflected by the plasma levels of hs-cTnT and cardiac isoprostanes, that significantly increased at 2 and 4 hr post-resuscitation ($p < 0.01$ vs. healthy rats, **Table 9** and **Figure 31**). Changes in plasma levels of these biomarkers of myocardial damage and oxidative stress were significantly inversely related to the concurrent changes in TRP and significantly directly related to changes in KYNA and 3-HAA (**Table 10**).

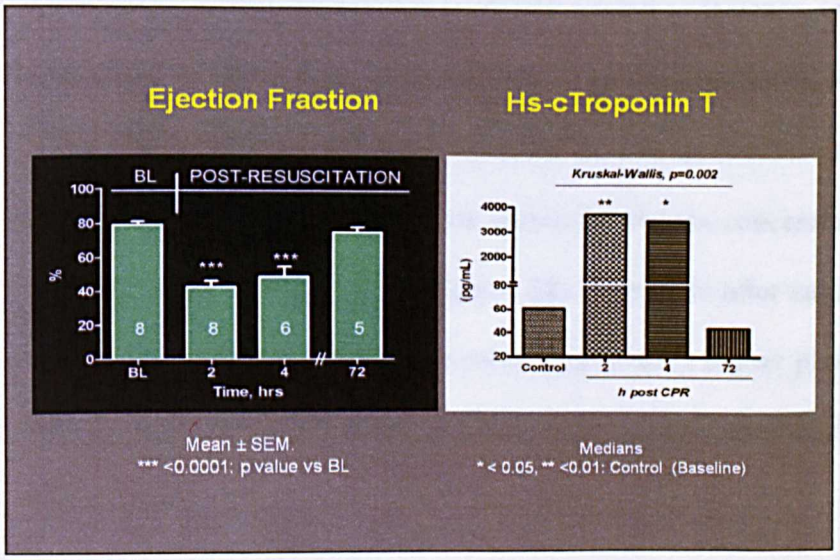


Figure 31. Left panel, post resuscitation LV ejection fraction and right panel, high sensitive troponin T, in control rats (or baseline) and at 2, 4, and 72 hr after resuscitation.

Table 10. Correlation between KP metabolites and hemodynamics, myocardial function, and biomarkers in resuscitated rats.

KP metabolite	Variable	r coefficient	p value
TRP	EF	0.55	0.005
	SV	0.53	0.011
	DT	0.699	0.000
	hs-cTnT	-0.501	0.017
	c-Isoprostanes	-0.516	0.014
KYNA	EF	-0.501	0.029
	SV	-0.558	0.016
	DT	-0.523	0.026
	hs-cTnT	0.641	0.004
3-HAA	EF	-0.611	0.003
	SV	-0.598	0.004
	DT	-0.470	0.031
	hs-cTnT	0.581	0.006
	c-Isoprostanes	0.523	0.015

KP, kynurenine pathway; TRP, tryptophan; KYNA, kynurenic acid; 3-HAA, 3-hydroxyanthranilic acid; EF, ejection fraction; CO, cardiac output; SV, stroke volume; DT, deceleration time; hs-cTnT, high sensitive cardiac troponin T; c-Isoprostanes, cardiac isoprostanes.

Pigs. Similarly to rats, plasma levels of TRP tended to decrease, while those of KYN tended to increase at 1 hr and 5 days post-resuscitation (p not significant, **Figure 32**). Plasma levels of KYNA and 3-HAA significantly increased at 1 hr post-resuscitation compared to baseline values ($p < 0.01$, **Figure 32**). These increased plasma concentrations of TRP's metabolites persisted 5 days later ($p < 0.05$, **Figure 32**). Five days after cardiac arrest, animals that had been subjected to hypothermia presented significantly higher plasma levels, approximately a 2-fold increase, of TRP, KYN, KYNA, and 3-HAA, compared to normothermic animals **(Figure 33)**.

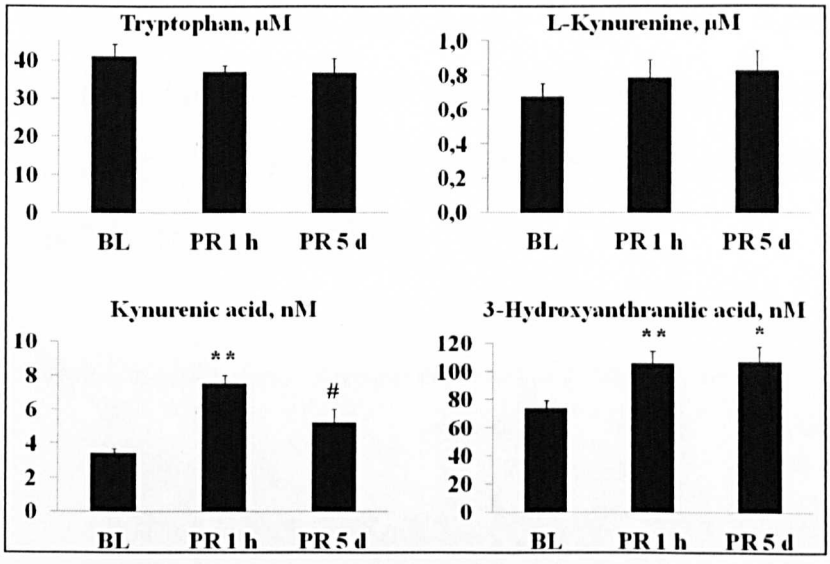


Figure 32. Kynurenine pathway activation in 10 pigs resuscitated from cardiac arrest. Plasma concentrations of trypthophan, L-kynurenine, kynurenic acid and 3-hydroxyanthranilic acid at baseline (BL) and after 1 hour (h), and 5 days (d) post-resuscitation (PR).
Data are reported as mean ± SEM.
* $p < 0.05$ and ** $p < 0.01$ vs. BL;
# $p < 0.05$ vs. PR 1 h.

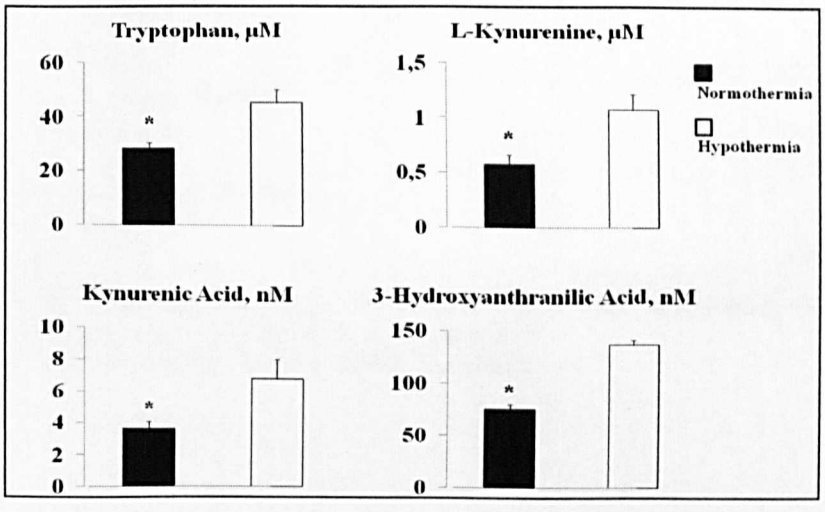


Figure 33. Kynurenine pathway activation and hypothermia in pigs, 5 days after resuscitation from cardiac arrest. Plasma concentrations of trypthophan, L-Kynurenine, kynurenic acid and 3-hydroxyanthranilic in pigs that had been subjected to hypothermia or normothermia after resuscitation.
Data are reported as mean ± SEM, (n = 5 pigs/group).
* $p < 0.01$.

Post-resuscitation myocardial dysfunction developed in each pig, as evidenced by the hemodynamic measurements in **Table 11**. More specifically, lower MAP and LV CO were observed at 1 and 4 hr post-resuscitation ($p < 0.01$ vs. baseline, **Table 11**). These decreases in MAP and CO were significantly inversely related to the increases in KYNA and 3-HAA (**Table 12**). Neurological deficit score at day 1, 3 and 5 post-resuscitation is reported in **Table 11**, together with cerebral histopathological scores, i.e. cortical and CA1 hippocampal lesion,

necrosis and astrogliosis. Early changes in 3-HAA were significantly directly related to the severity of neurological deficit score (**Figure 34**) and to the histological alterations (**Table 12**). Overall changes in TRP were significantly inversely related to the severity of hippocampal lesions (**Table 12**).

Table 11. Hemodynamics, functional and histological outcome, and biomarkers in resuscitated pigs.

	Baseline	**PR 1 hr**	**PR 4 hr**
MAP, mmHg	121 ± 4	76 ± 5**	86 ± 4**
CO, mL/min	5.8 ± 0.3	2.9 ± 0.3**	3.4 ± 0.2**
	PR 1 day (score)	**PR 3 days** (score)	**PR 5 days** (score)
NDS	66.5 ± 5.3	39.5 ± 10.3	41 ± 12.6
Brain lesion Cortex CA1			 7.7 ± 1 6.5 ± 0.7
Brain necrosis Cortex CA1			 1.5 ± 0.2 2.4 ± 0.4
Brain astrogliosis Cortex CA1			 1.4 ± 0.2 1.8 ± 0.25

MAP, mean arterial pressure; CO, cardiac output; NDS, neurological deficit score; CA1, CA1 hippocampal sector; PR, post-resuscitation; N.A., not available.
** $p < 0.01$ and * $p < 0.05$ vs. Healthy or Baseline.

Table 12. Correlation between KP metabolites and hemodynamics, myocardial function, and brain histology in resuscitated pigs.

KP metabolite	**Variable**	**r coefficient**	**p value**
TRP	CA1 lesions	-0.728	0.017
KYNA	MAP	-0.799	0.000
	CO	-0.742	0.000
3-HAA	MAP	-0.561	0.016
	CO	-0.729	0.001
	CA1 necrosis	0.792	0.006
	Cortical astrogliosis	0.778	0.008
	NDS	0.744	0.014

KP, kynurenine pathway; TRP, tryptophan; KYNA, kynurenic acid; 3-HAA, 3-hydroxyanthranilic acid; CO, cardiac output; MAP, mean arterial pressure; CA1, CA1 hippocampal area; NDS, neurological deficit score.

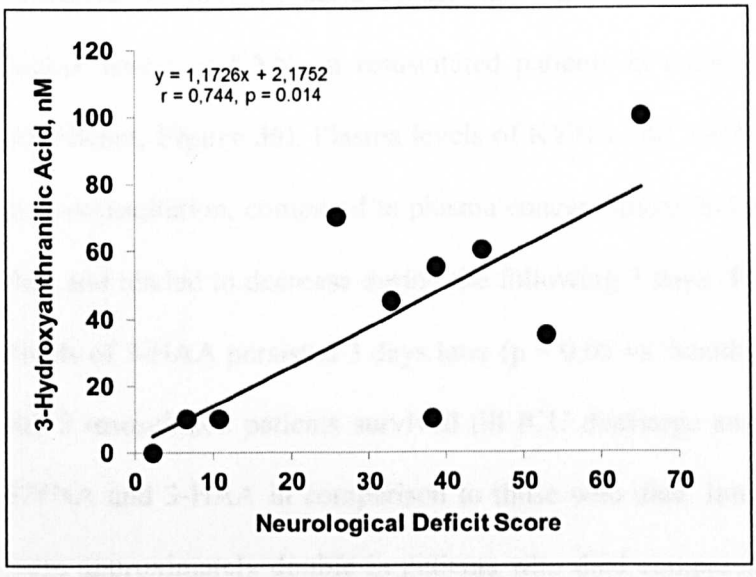


Figure 34. Correlation between early changes in plasma level of 3-hydroxyanthranilic acid and 3 day neurological deficit score in pigs resuscitated from cardiac arrest.

As expected animals treated with xenon showed a significantly better NDS through postoperative days 1 to 3. On the first day following CPR, significantly more animals in the control group were not able to stand up when compared with the animals treated with 1 hr of xenon (7 of 8 vs. 1 of 8; $p < 0.05$). Strikingly, in animals subjected to this known neuroprotective gas, post-resuscitation decreases in TRP and increases in 3-HAA were not observed (p, not significant, **Figure 35**). On the contrary, KYNA significantly increased 5 day post-resuscitation ($p< 0.05$ vs. baseline level, **Figure 35**).

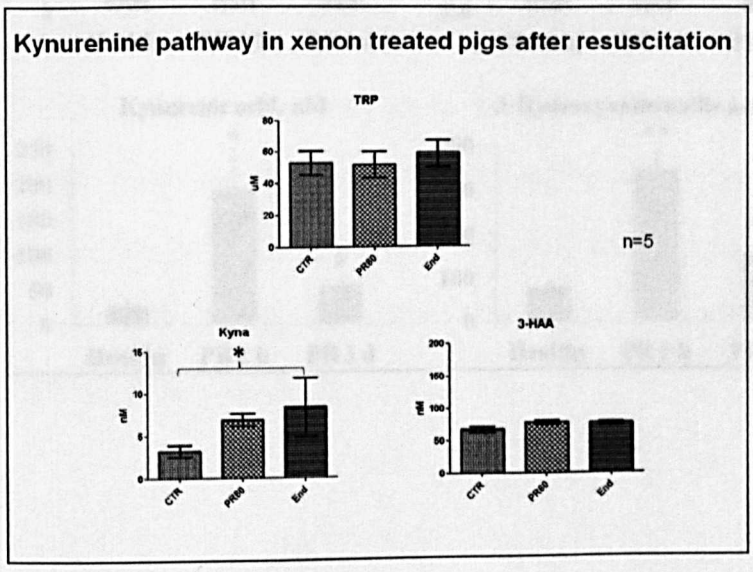


Figure 35. Kynurenine pathway activation in pigs resuscitated from cardiac arrest. Plasma concentrations of trypthophan (TRP), kynurenic acid (KYNA) and 3-hydroxyanthranilic acid (3-HAA) at baseline (CTR) and after 1 hr (PR 60), and 5 days (end) post-resuscitation (PR).
Data are reported as mean ± SEM.
* $p < 0.05$ and vs. baseline (CRT).

Humans. Similarly to rats and pigs, there was a trend towards lower plasma levels of TRP and higher levels of KYN, in resuscitated patients in comparison to healthy volunteers (p not significant, **Figure 36**). Plasma levels of KYNA and 3-HAA were significantly higher at 1 hr post-resuscitation, compared to plasma concentrations in healthy volunteers ($p < 0.01$, **Figure 36**), and tended to decrease during the following 3 days (**Figure 36**). However, higher plasma levels of 3-HAA persisted 3 days later ($p < 0.05$ vs. healthy volunteers, **Figure 36**). Only 2 of the 5 resuscitated patients survived till ICU discharge and presented lower plasma levels of KYNA and 3-HAA in comparison to those who died. Interestingly, plasma levels of 3-HAA were approximately double in patients who died compared to those who survived (190 ± 12 nM vs. 102 ± 12 nM).

Post-resuscitation circulating levels of pro-inflammatory cytokines, PCT, and CRP are reported in **Table 13**.

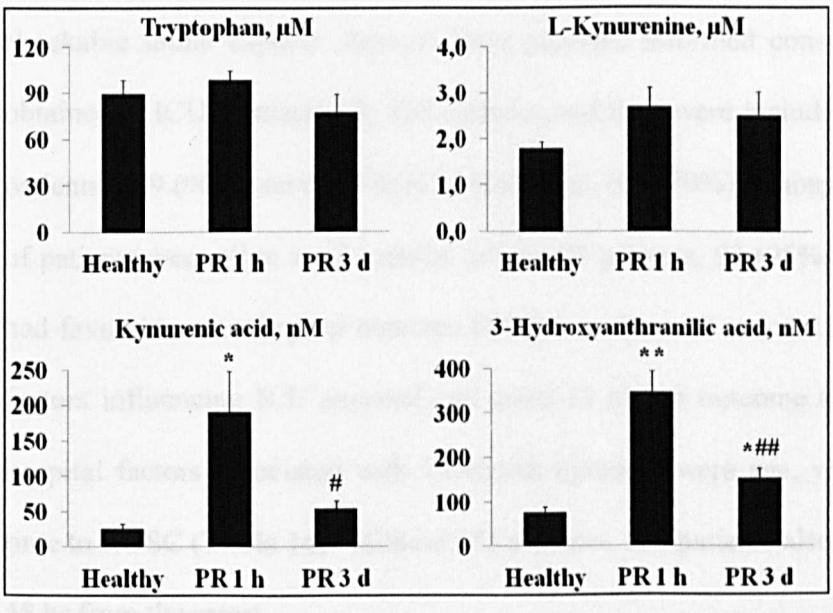


Figure 36. Kynurenine pathway activation in men resuscitated from cardiac arrest. Plasma concentrations of trypthophan, L-kynurenine, kynurenic acid and 3-hydroxyanthranilic acid in healthy volunteers and in men after 1 hour (h), and 3 days (d) post -resuscitation (PR). Data are reported as mean ± SEM.
* $p < 0.05$ and ** $p < 0.01$ vs. BL;
# $p < 0.05$ and ## $p < 0.01$ vs. PR 1 h.

Table 13. Pro-inflammatory biomarkers in men resuscitated from cardiac arrest.

	PR 1 hr	PR 3 days
Procalcitonin, mg/mL	0.6 ± 0.2	2.6 ± 2.1
IL-6, ng/mL	92 ± 39	63 ± 22
IL-8, ng/mL	51 ± 18	18 ± 2
TNFa, ng/mL	11 ± 1	11 ± 3
MIF, ng/mL	549 ± 42	197 ± 63*
CRP, ng/mL	12 ± 9	139 ± 21*

IL, interleukin; TNFa, tumor necrosis factor alpha; MIF, macrophage inhibitor factor; CRP, C-reactive protein; PR, post resuscitation.
* $p < 0.01$ vs. PR 1 hr.

- **STUDY 3: Validation of KP activation after cardiac arrest in a large cohort of out-of-hospital cardiac arrest patients**

The FINNIRESUSCI study *(Vaahersalo 2013)* included 548 patients of which 311 had a shockable initial rhythm. Among these patients, informed consent for blood sampling was obtained at ICU admission in 155 patients, and they were included in the study. Of these 155 patients, 139 (90%) survived to ICU discharge, 109 (70%) to hospital discharge, and 97 (63%) of patients were alive at 12 months. Of the 97 patients, 92 (95%, or 59% of the 155 patients) had favorable neurological outcome (CPC 1 or 2) at 12 months. Baseline characteristics and factors influencing ICU survival and good 12 month outcome are shown in **Table 14**. Pre-hospital factors associated with 12-month outcome were age, witnessed arrest (yes/no) and time to ROSC (**Table 14**). Of these 155 patients, 134 patients also had blood samples drawn at 48 hr from the arrest.

Table 14. Baseline characteristics and factors at resuscitation indexed by comparisons between ICU survivors and non-survivors and those with good and poor outcome at 12 months.

Characteristic	ICU death (n=16)	ICU survival (n=139)	Poor outcome at 12 months (n=63)	Good outcome at 12 months (n=92)
Age, median (IQR)	68(63-76)	62(57-72)*	69(62-75)	61(55-67)§§
Gender (male), n(%)	14(88%)	118(85%)	57(90%)	75(82%)
Witnessed cardiac arrest, n(%)	13(81%)	131(94%)*	55(87%)	89(97%)§
Bystander initiated life support, n(%)	11(69%)	94(67%)	41(65%)	64(70%)
Adrenaline used, n(%)	16(100%)	78(56%)**	54(86%)	40(44%)§§
Time to ROSC, min median(IQR)	24(21-31)	20(13-28)**	25(21-32)	17(11-23)§§
Therapeutic hypothermia, n(%)	14(88%)	117(84%)	55(87%)	76(83%)
SAPS II score, median(IQR)	74(64-83)	55(37-66)**	66(57-72)	47(34-60)§§

SAPS II = Simplified acute physiology score; IQR=Interquartile range.
* $p < 0.05$ and ** $p < 0.01$ vs. ICU death; § $p < 0.05$ and §§ $p < 0.01$ vs. Poor outcome at 12 months.

KP metabolites and factors related to resuscitation

The levels of TRP and KP metabolites in the 10 healthy volunteers, matched for age and gender, were comparable to data reported in literature (TRP: 74.3 μM [61.1-88.0], KYN: 2.4 μM [2.2-3.0], KYNA: 49.0 nM [35.5-84.3] and 3-HAA: 25.0 nM [29-42]) (*Darlington 2007, Mackay 2006*). At ICU admission, the levels of KYN and KYN/TRP were higher in older patients (**Table 15**). All metabolites, but TRP, were also significantly higher in patients with longer time to ROSC (**Table 15**). Patients awake at ICU admission had higher TRP levels, but no difference was seen regarding the other metabolites (**Table 15**). In a linear regression model, the inclusion of age, gender, whether the cardiac arrest was witnessed or not, administration of BLS, and time to ROSC was an independent predictor of admission levels of KYN (β=0.028 95% CI 0.008-0.047, p=0.005), KYNA (β 2.12 95% CI 0.299-3.933, p=0.023), 3-HAA (β 0.541 95% CI 0.125-0.956, p=0.011), KYN/TRP ratio (β=0.001, 95% CI 0.000-0.001, p=0.015), but not TRP (β=-0.005 95% CI -0.26-0.15, p=0.617).

Correlations between KP metabolites and severity of shock

Levels of all metabolites except 3-HAA at ICU admission were associated with the subsequent 24 hr SAPS II systolic blood pressure scores, with significantly higher levels in patients who had lower systolic blood pressure (**Table 15**). Levels of all metabolites at ICU admission were also associated with SAPS II bicarbonate scores during the following 24 hours (**Table 15**).

Table 15. KP indexed by duration of patient characteristics, duration of cardiac arrest and patient outcome.

Variable	TRP μM	KYN μM	KYN/TRP ratio	KYNA nM	3-HAA nM
Admission levels					
Age, year					
53 (18-59)	43.2 (34.5-57.9)	2.1 (1.5-2.9)	0.05 (0.04-0.07)	61.2 (35.0-103.3)	24.7 (18.1-41.8)
63 (60-68)	41.7 (34.4-48.4)	2.3 (1.8-3.1)	0.06 (0.04-0.07)	53.9 (38.4-90.6)	24.2 (16.6-44.4)
75 (69-88)	43.2 (32.9-55.4)	2.8 (2.1-3.8)	0.06 (0.05-0.09)	68.9 (48.3-113.0)	26.7 (16.8-41.2)
p-value	0.620	0.005	0.001	0.412	0.874
Time to ROSC, min					
1-16	43.7 (37.7-56.1)	2.2 (1.5-2.9)	0.05 (0.04-0.06)	53.0 (35.1-77.9)	20.9 (13.3-32.0)
17-24	40.8 (31.3-54.9)	2.6 (1.7-3.3)	0.06 (0.05-0.09)	64.6 (38.5-120.5)	28.6 (18.2-42.4)
25-57	41.7 (33.3-51.5)	2.6 (1.9-3.2)	0.07 (0.05-0.08)	66.7 (49.8-129.7)	28.1 (19.4-48.5)
p-value	0.339	0.066	0.0007	0.043	0.009
Lowest 24-hour systolic blood pressure, mmHg					
>100	52.3 (33.2-63.7)	2.3 (1.7-3.2)	0.05 (0.04-0.07)	43.5 (43.5-46.9)	21.3 (14.6-44.0)
70-99	41.6 (33.9-50.9)	2.4 (1.7-3.0)	0.06 (0.04-0.07)	58.0 (39.7-88.3)	24.7 (17.3-39.7)
<70	47.9 (40.4-60.9)	3.4 (2.9-4.8)	0.07 (0.06-0.1)	141.6 (58.4-478.1)	44.6 (19.0-116.3)
p-value	0.144	0.004	0.025	0.035	0.174
24-hour HCO3, mEq/l					
>20	43.0 (34.5-55.5)	2.1 (1.6-2.9)	0.05 (0.04-0.07)	52.6 (38.1-77.6)	23.4 (16.0-36.2)
15-19	41.3 (33.1-50.9)	2.6 (1.9-3.4)	0.06 (0.05-0.08)	64.6 (39.9-117.6)	24.5 (18.4-41.3)
<15	44.8 (41.6-57.4)	3.2 (2.3-4.3)	0.07 (0.05-0.1)	191 (85.3-471.4)	77.5 (39.6-120.6)
p-value	0.288	0.003	0.001	0.001	0.001
Outcome at 12 months					
Good	42.9 (34.7-55.3)	2.1 (1.7-2.9)	0.048 (0.040-0.073)	54.6 (38.6-86.9)	22.4 (16.3-36.9)
Poor	41.7 (33.8-51.6)	2.7 (2.2-3.5)	0.065 (0.055-0.087)	67.6 (43.1-160.4)	29.9 (18.2-45.4)
p-value	0.956	<0.001	<0.001	0.043	0.047

[1] Wilcoxon signed rank test for paired data; KP metabolites are reported as median(IQR).

KP metabolites at ICU admission and outcome

All KP metabolites, but not TRP, correlated with ICU mortality (**Figure 37**) and outcome at 12-months (**Table 15** and **Figure 38**). KP metabolite levels were significantly higher in patients who died in the ICU or prior to hospital discharge compared to those who survived (**Figure 37** and **Table 15**), and in patients with a poor outcome at 12 months compared to those with a good outcome (**Table 15** and **Figure 38**). The AUC for the prediction of ICU mortality was 0.813 (95% CI 0.724-0.903, p<0.001) for KYN, 0.780 (95% CI 0.660-0.899, p<0.001) for KYNA, 0.757 (95% CI 0.625-0.888, p<0.001) for 3-HAA and 0.766 (95% CI 0.662-0.869, p<0.001) for KYN/TRP ratio.

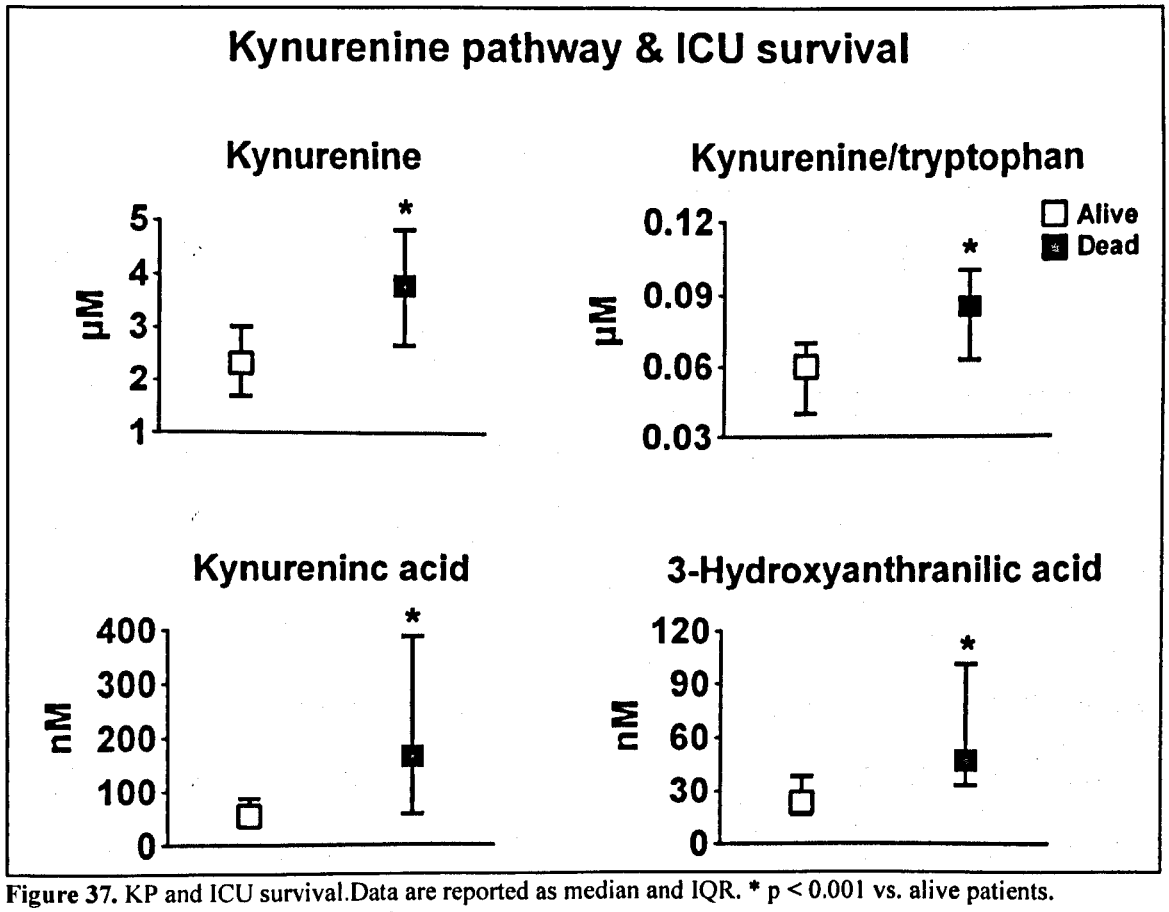


Figure 37. KP and ICU survival.Data are reported as median and IQR. * p < 0.001 vs. alive patients.

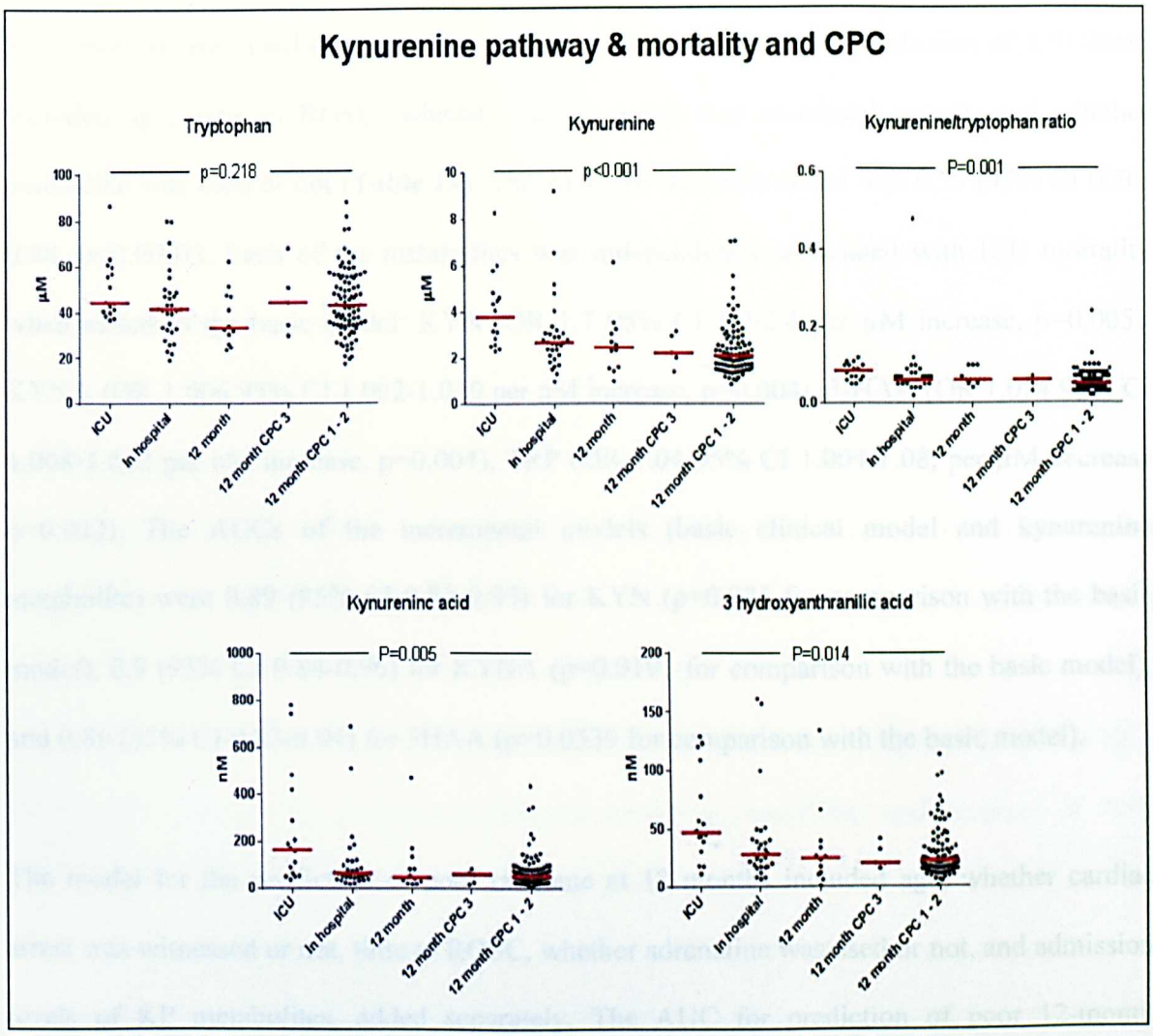


Figure 38. KP and 12-month mortality and CPC. Data are reported as scatter plot and median.

The AUC values for prediction of poor 12-month outcome based on metabolite levels on admission were 0.665 (95% CI 0.578-0.752, p<0.001) for KYN, 0.598 (95% CI 0.503-0.688, p=0.043) for KYNA, 0.594 (0.502-0.686, p=0.047) for 3-HAA and 0.674 (95% CI 0.590-0.759, p<0.001) for the KYN/TRP ratio.

We developed multivariable models for the prediction of ICU mortality and for poor outcome at 12 months from cardiac arrest. The (basic) clinical model for the prediction of ICU death included age, time to ROSC, whether cardiac arrest was witnessed or not, and whether adrenaline was used or not (**Table 14**). The AUC for the basic model was 0.79 (95% CI 0.70-0.88, p<0.0001). Each of the metabolites was independently associated with ICU mortality when added to the basic model: KYN (OR 1.7 95% CI 1.2-2.4 per μM increase, p=0.005), KYNA (OR 1.006 95% CI 1.002-1.010 per nM increase, p=0.004), 3-HAA (OR 1.024 95% CI 1.008-1.042 per nM increase, p=0.004), TRP (OR 1.04 95% CI 1.004-1.08, per μM decrease p=0.032). The AUCs of the incremental models (basic clinical model and kynurenine metabolite) were 0.89 (95% CI 0.83-0.95) for KYN (p=0.025 for comparison with the basic model), 0.9 (95% CI 0.84-0.96) for KYNA (p=0.0191 for comparison with the basic model), and 0.89 (95% CI 0.83-0.94) for 3HAA (p=0.0339 for comparison with the basic model).

The model for the prediction of poor outcome at 12 months included age, whether cardiac arrest was witnessed or not, time to ROSC, whether adrenaline was used or not, and admission levels of KP metabolites, added separately. The AUC for prediction of poor 12-month outcome was 0.84 (95% CI 0.78-0.91) in the basic model. Higher levels of KYNA (OR 1.004 95% CI 1.001-1.007 per nM increase, p=0.038) and 3-HAA (OR 1.015 95% CI 1.010-1.030, per nM increase, p=0.043) correlated with poor outcome at 12 months. The AUC for prediction of poor outcome was 0.86 (95% CI 0.79-0.92) with the addition of KYNA into the model (p=0.18 for comparison with the basic model) and 0.86 with addition of 3-HAA (p=0.13 for comparison with the basic model).

- ***ECG-DERIVED BIOMARKERS:***

- **STUDY 4: AMSA evaluation in 609 VF patients in the United States**

A total of 1260 DFs, including 578 first attempts and 682 subsequent ones from 609 patients, were included in the analyses.

All DF attempts

Among all 1260 DFs, 316 were successful (25.1%), while 944 were unsuccessful (74.9%). AMSA was significantly higher prior to a successful DF than prior to an unsuccessful one (15.6 ± 0.6 vs. 7.97 ± 0.2 mV-Hz, $p<0.0001$, **Figure 39**).

Using the intersection of sensitivity, specificity, and accuracy curves (**Figure 40A**), an AMSA threshold of 9.8 mV-Hz, provided a balanced sensitivity, specificity and accuracy of 78%, with a NPV of 91% and a PPV of 54%. An AMSA threshold of 14 mV-Hz provided the highest accuracy (80%) in predicting DF outcome, with a PPV of 61% and a specificity of 90%. Higher AMSA thresholds were associated with further increases in PPV and specificity. An AMSA threshold of 17 mV-Hz resulted in the highest PPV (67%) in predicting DF success.

For low AMSA thresholds, the majority of unsuccessful DFs were correctly predicted with high sensitivity and NPV (**Figure 40A**). Using AMSA < 7 mV-Hz as cutoff, more than 42% of unsuccessful DFs might have been avoided with a NPV > 97% (**Figure 40A** and **Figure 41**). Lower thresholds further improved NPV.

Area under the ROC curve was 0.84 ($p<0.0001$, **Figure 40B**).

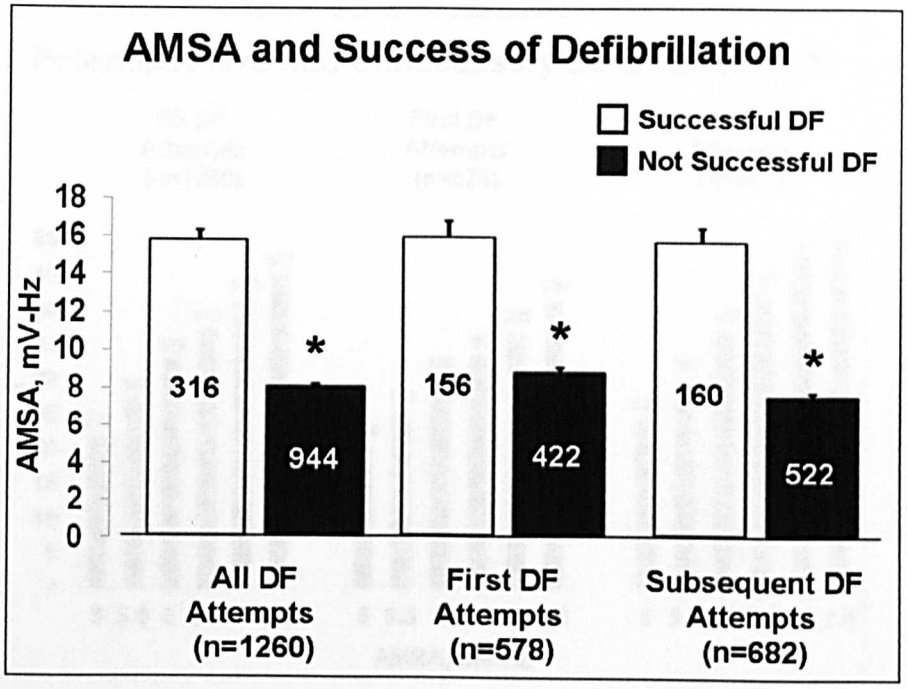


Figure 39. AMSA values for successful and not successful DF for all, first and subsequent attempts. Number of attempts are reported inside bars.
* $p<0.0001$ between successful and not successful DFs.

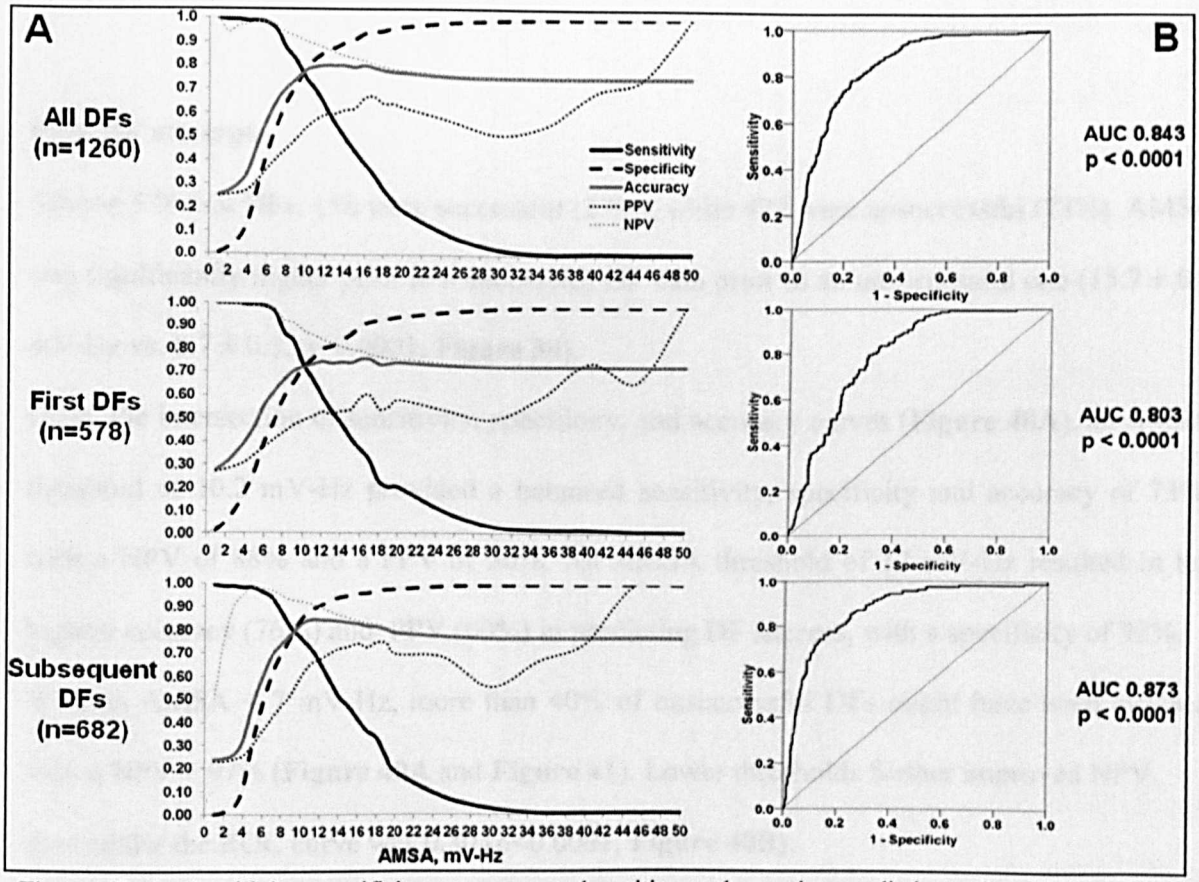


Figure 40. A: Sensitivity, specificity, accuracy, and positive and negative predictive value (PPV and NPV) curves for different AMSA values, in all DFs, first DFs, and subsequent DFs.
B: ROC curves for AMSA and DF outcome prediction for all, first, and subsequent DFs.

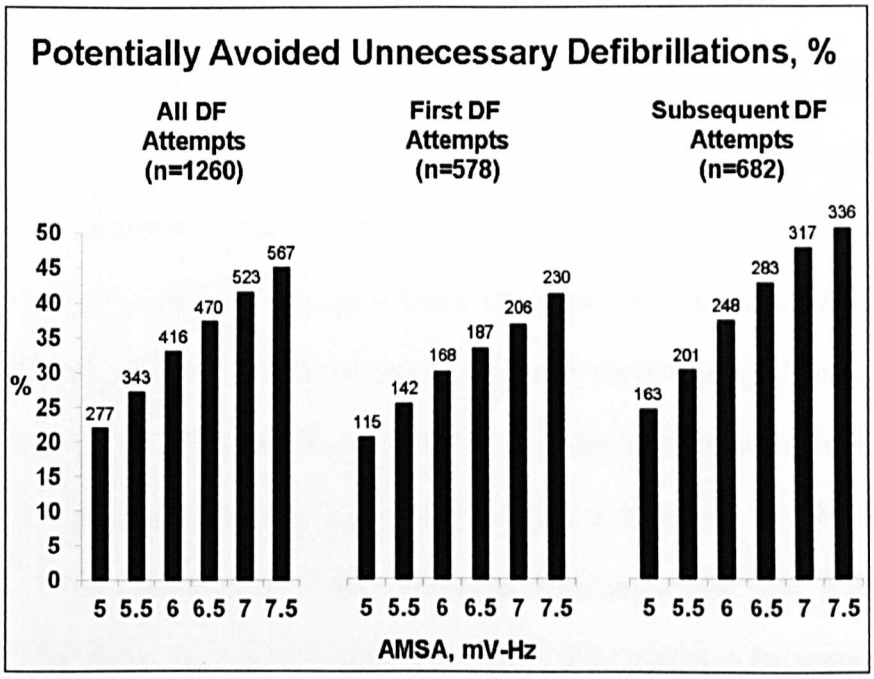


Figure 41. Percentage of potentially avoided unnecessary DFs for different low AMSA thresholds. Numbers on the top of bars represent the correctly predicted unsuccessful DFs.

First DF attempts

Among 578 first DFs, 156 were successful (27%), while 422 were unsuccessful (73%). AMSA was significantly higher prior to a successful DF than prior to an unsuccessful one (15.7 ± 0.9 mV-Hz vs. 8.7 ± 0.3, $p<0.0001$, **Figure 39**).

Using the intersection of sensitivity, specificity, and accuracy curves (**Figure 40A**), an AMSA threshold of 10.2 mV-Hz provided a balanced sensitivity, specificity and accuracy of 73%, with a NPV of 88% and a PPV of 50%. An AMSA threshold of 17 mV-Hz resulted in the highest accuracy (76%) and PPV (60%) in predicting DF success, with a specificity of 92%. With an AMSA < 7 mV-Hz, more than 40% of unsuccessful DFs might have been avoided with a NPV > 97% (**Figure 40A** and **Figure 41**). Lower thresholds further improved NPV. Area under the ROC curve was 0.80 ($p<0.0001$, **Figure 40B**).

Subsequent DF attempts

Among 682 subsequent DFs, 160 were successful (23.5%), while 522 were unsuccessful (76.5%). AMSA was significantly higher prior to a successful DF than prior to an unsuccessful one (15.5 ± 0.8 mV-Hz, $p<0.0001$, **Figure 39**).

Using the intersection of sensitivity, specificity, and accuracy curves (**Figure 40A**), an AMSA threshold of 9.2 mV-Hz provided a balanced sensitivity, specificity and accuracy of 79%, with a NPV of 93% and a PPV of 54%. An AMSA threshold of 14 mV-Hz resulted in the highest accuracy (84%) in predicting DF success, with a PPV of 72% and a specificity of 94%. Further increases in AMSA threshold were associated with further increases in PPV and specificity. An AMSA threshold of 18 mV-Hz resulted in the highest PPV (75%) in predicting DF success.

With an AMSA < 7 mV-Hz, more than 46% of unsuccessful DFs might have been avoided with a NPV > 97% (**Figure 40A** and **Figure 41**). Lower thresholds further improved NPV.

Area under the ROC curve was 0.87 ($p<0.0001$, **Figure 40B**).

DF attempts for recurrent and refractory VF

Among 682 subsequent DF attempts, 139 were delivered for recurrent VF, while 543 were for refractory VFs. Among 139 DFs delivered for recurrent VF, 110 were successful (79.1%), while of 543 DFs delivered for refractory VF, only 50 were successful (9.2%) (**Table 16**). AMSA was significantly higher in recurrent VF than in refractory VF (16.2 ± 0.9 vs. 7.6 ± 0.2 mV-Hz, $p<0.0001$, **Table 16**). In the instance of recurrent VF, there was no significant difference in AMSA prior to a successful DF compared to that prior to an unsuccessful one (16.8 vs. 13.8 mV-Hz, **Table 16**). For refractory VFs, AMSA was significantly higher prior to a successful DF than prior to an unsuccessful one (12.7 ± 1 vs. 7 ± 0.2 mV-Hz, $p<0.0001$,

Table 16). AMSA was accurate for predicting DF outcome only in the instance of refractory VF (**Figure 42**).

In the instance of refractory VF, the intersection of sensitivity, specificity, and accuracy curves provided an AMSA threshold of 8.5 mV-Hz accounting for a balanced sensitivity, specificity and accuracy of 77% (**Figure 42A**). An AMSA threshold of 17 mV-Hz resulted in peak accuracy (91%) and peak PPV (50%), with a specificity of 97%.

Area under the ROC curve was 0.81 ($p<0.0001$) for refractory VF, and 0.65 for recurrent VF ($p=0.012$, **Figure 42B**).

Table 16. AMSA prior to defibrillation for refractory and recurrent VF

	Refractory VF (n=543)	**Recurrent VF** (n=139)
Mean AMSA, mV-Hz	7.6 ± 0.2	16.2 ± 0.9*
AMSA prior to successful DFs, mV-Hz	12.7 ± 1	16.8 ± 1
AMSA prior to failing DFs, mV-Hz	7.0 ± 0.2 #	13.8 ± 1.8
Successful DFs, % (n)	9.2 (50/543)	79.1 (110/139)

DFs, defibrillation attempts; VF, ventricular fibrillation.
Mean ± SEM; * $p < 0.0001$ vs. refractory VF; # $p < 0.0001$ vs. successful DFs

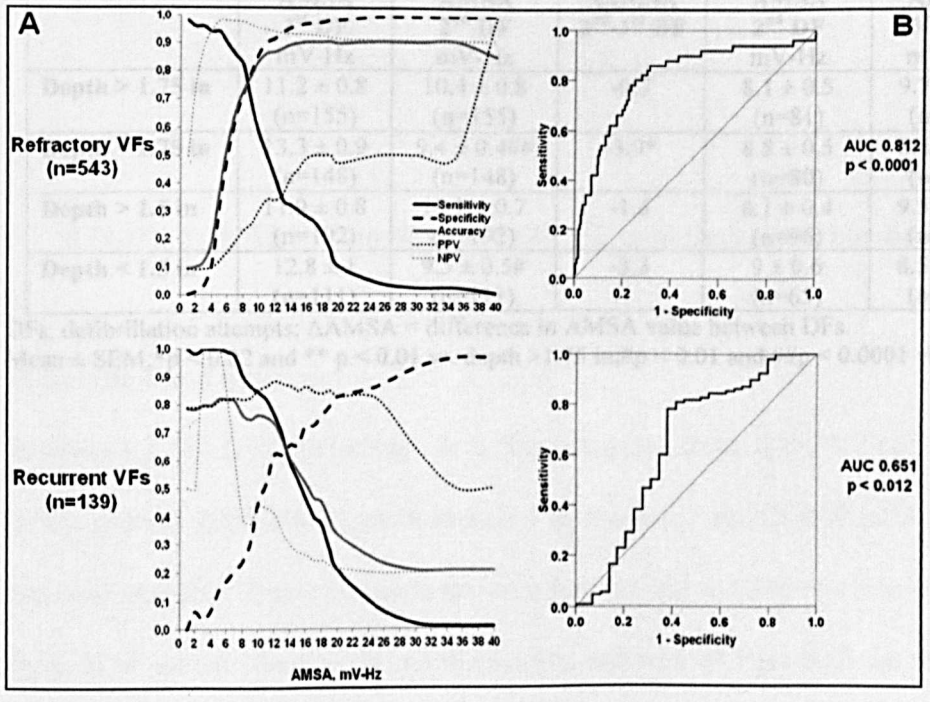


Figure 42.
A: Sensitivity, specificity, accuracy, and positive and negative predictive value (PPV and NPV) curves for different AMSA values, in DFs delivered for refractory and recurrent VFs.
B: ROC curve for AMSA and DF outcome prediction for DFs delivered for refractory and recurrent VFs.

Influence of CC depth and interruptions on AMSA

When CC depth was <1.75 in, AMSA decreased by 3.9 mV-Hz between the first and the second DF and further decreased between the second and the third attempt (**Table 17**, $p<0.001$). By contrast, when CC depth was >1.75 in, AMSA was equivalent for the first and the second DF ($p=0.47$) and then increased by 1.7 mV-Hz between the second and the third attempt. A similar but less pronounced trend was observed with a CC depth cutoff at 1.5 in (**Table 17**).

AMSA significantly decreased during the 16 sec pre-DF CC interruption for AED rhythm analyses and capacitor charge ($p<0.01$, **Figure 43**). AMSA decreased even more during CC interruptions prior to first DF ($p<0.01$). In subsequent DFs, AMSA decrease during CC interruptions was less pronounced ($p<0.05$ only after 12 sec CC interruption).

Table 17. AMSA value changes among the initial 3 defibrillation attempts in relationship to chest compression depth.

	AMSA 1st DF mV-Hz	AMSA 2nd DF mV-Hz	ΔAMSA 2nd-1st DF	AMSA 2nd DF mV-Hz	AMSA 3rd DF mV-Hz	ΔAMSA 3rd-2nd DF
Depth > 1.75 in	11.2 ± 0.8 (n=155)	10.4 ± 0.8 (n=155)	-0.7	8.1 ± 0.5 (n=81)	9.7 ± 0.8 (n=81)	+1.7
Depth < 1.75 in	13.3 ± 0.9 (n=148)	9.4 ± 0.4## (n=148)	-3.9*	8.8 ± 0.5 (n=80)	8.4 ± 0.5 (n=80)	-0.4**
Depth > 1.5 in	11.9 ± 0.8 (n=192)	10.1 ± 0.7 (n=192)	-1.6	8.1 ± 0.4 (n=96)	9.5 ± 0.7 (n=96)	+1.5
Depth < 1.5 in	12.8 ± 1 (n=111)	9.5 ± 0.5# (n=111)	-3.3	9 ± 0.6 (n=65)	8.5 ± 0.6 (n=65)	-0.6**

DFs, defibrillation attempts; ΔAMSA = difference in AMSA value between DFs.
Mean ± SEM;*p < 0.02 and ** p < 0.01 vs. depth >1.75 in;#p < 0.01 and ##p < 0.0001 vs. 1st DF.

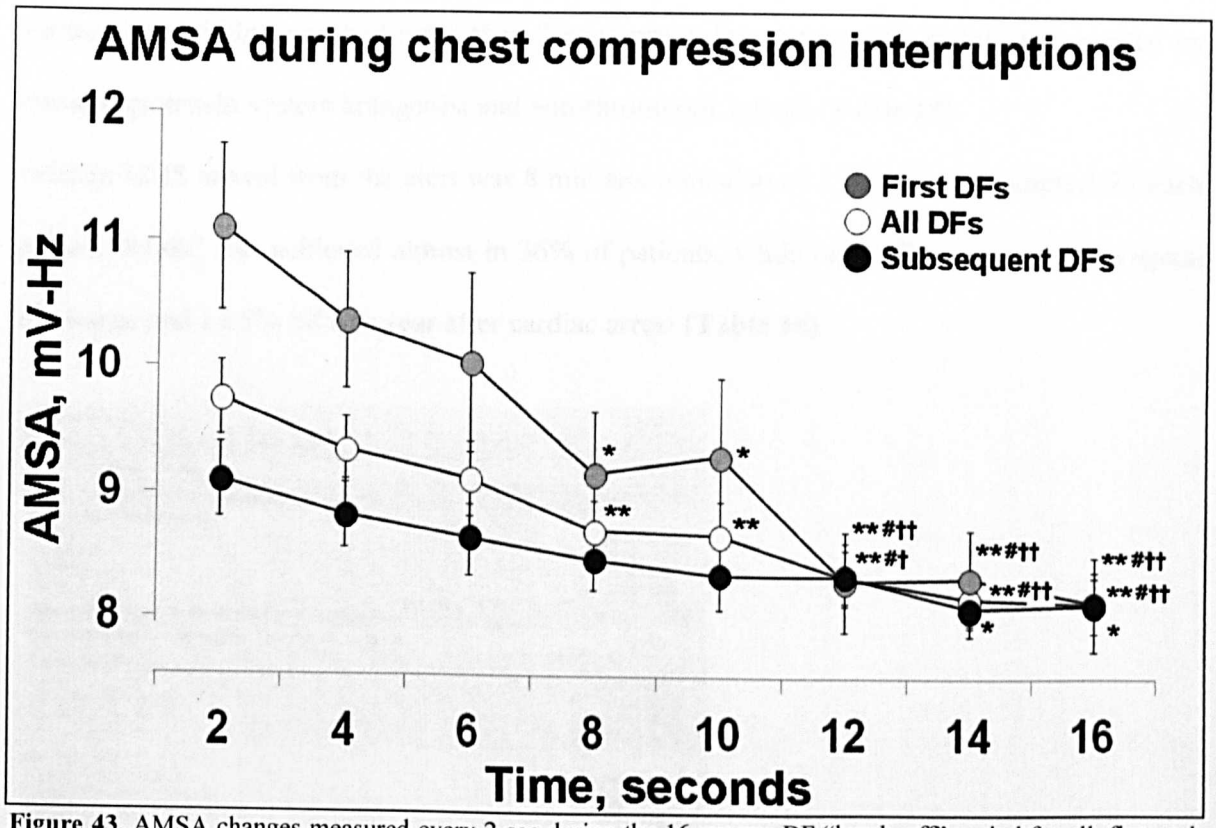


Figure 43. AMSA changes measured every 2 sec during the 16 sec pre-DF "hands off" period for all, first and subsequent DFs.
* $p < 0.05$ and ** $p < 0.01$ vs. 2 sec;
# $p < 0.01$ vs. 4 sec;
† $p < 0.05$ and †† $p < 0.01$ vs. 6 sec.

- **STUDY 5: AMSA evaluation in 1.617 VF patients in Lombardia Region, Italy**

Population and outcome

A total of 2.447 DFs, including 1.050 first attempts, from 1.050 VF patients in the derivation group (period 2008-2009), were included in the analyses. Of 860 patients among the 1.050, baseline characteristics and outcome were known and are summarized in **Table 18**. Seventy-three % of patients were male and the median age was 69.5 years. In more than 92% of cardiac

arrests, the EMS was alerted for a medical cause. More than 50% of patients presented a cardiac comorbidity and had more than 2 concurrent drug treatments, mainly represented by renin-angiotensin system antagonist and anti-thrombotic agents (**Table 18**).

Median EMS arrival from the alert was 8 min and a median of 2 DFs were attempted for each patient. ROSC was achieved almost in 36% of patients, while only 18% survived till hospital discharge and 14.5% till one year after cardiac arrest (**Table 18**).

Patients (total), N	1.050
Patients (with known outcome), N (%)	860 (81.9)
Gender (male), N (%)	629 (73.14)
Age in years, median ($q_{0.25}$-$q_{0.75}$)	69.5 (59-78)
Cause EMS alert, N (%)	
Medical	799 (92.91)
Traumatic	32 (3.72)
Tumoral	3 (0.35)
Other	26 (3.02)
EMS arrival time in minutes, median ($q_{0.25}$-$q_{0.75}$)	8 (6.1-10.7)
N of defibrillation attempts, median ($q_{0.25}$-$q_{0.75}$)	2 (1-3)
Outcome, N (%)	
ROSC	308 (35.81)
Hospital discharge	160 (18.6)
Six-month survival	139 (16.2)
One-year survival	125 (14.5)
Comorbidities, N (%)	
Myocardial infarction	259 (30.12)
Congestive heart failure	220 (25.58)
Peripheral vascular disease	88 (10.23)
Cerebrovascular disease	122 (14.19)
Chronic pulmonary disease	10 (1.16)
Diabetes	119 (13.84)
Liver disease	51 (5.93)
Renal disease	21 (2.44)
Cancer	70 (8.14)
Others	51 (5.93)
Drug Treatment, N (%)	
Cardiac therapy	229 (26.63)
Antithrombotic drugs	350 (40.70)
Antihypertensive drugs	37 (4.30)
Beta blocker drugs	207 (24.07)
Calcium channel blockers	190 (22.09)
Renin-angiotensin system antagonists	444 (51.63)
Lipid modifying agents	205 (23.84)
Selective $beta_2$ adrenoreceptor agonists	15 (1.74)
Concurrent drug treatments, N (%)	
0	256 (29.77)
1	133 (15.47)
2	142 (16.51)
3	144 (16.74)
4	114 (13.26)
≥5 (max 7)	71 (8.26)

Table 18. Baseline population and outcome

AMSA and other ECG-derived parameters

Mean values of AMSA, RMS, PF, MDF, and MNF for all, first, subsequent and last DF attempts are reported in **Table 19**. No differences in AMSA and RMS values were observed between the first and the subsequent DFs. MDF and MNF, instead significantly increased

between the first and the subsequent attempt (**Table 19**). More specifically, average AMSA values were 8.39, 8.27, and 8.48 mV-Hz, for all, first, and subsequent DFs respectively. No differences were observed between values calculated in all 1.050 patients vs. those calculated in the 860 ones with known outcome.

Table 19. ECG variables

	Defibrillation attempt	AMSA (mV-Hz)	RMS (mV)	PF (Hz)	MDF (Hz)	MNF (Hz)
All patients (1050)	All	8.39 ± 4.88	0.13 ± 0.09	3.77 ± 1.709	4.48 ± 1.421	7.59 ± 1.568
	First	8.27 ± 4.95	0.14 ± 0.09	3.58 ± 1.598*	4.30 ± 1.335*	7.30 ± 1.487*
	Subsequent	8.48 ± 4.83	0.13 ± 0.08	3.90 ± 1.775	4.60 ± 1.469	7.80 ± 1.593
	Last	9.04 ± 5.18	0.15 ± 0.09	3.88 ± 1.685	4.52 ± 1.435	7.43 ± 1.439
Patients with known outcome (860)	All	8.31 ± 4.81	0.13 ± 0.09	3.75 ± 1.713	4.50 ± 1.429	7.62 ± 1.558
	First	8.16 ± 4.89	0.14 ± 0.09	3.54 ± 1.593*	4.31 ± 1.353*	7.32 ± 1.495*
	Subsequent	8.42 ± 4.75	0.13 ± 0.08	3.91 ± 1.779	4.64 ± 1.466	7.83 ± 1.566
	Last	9.01 ± 5.07	0.15 ± 0.09	3.83 ± 1.675	4.53 ± 1.430	7.45 ± 1.418

Mean ± SD; * p<0.001 vs. subsequent defibrillation attempts

Using linear regression models, age, gender, EMS arrival time, presence of a cardiac, pulmonary, and renal disease, diabetes, anti-thrombotic, anti-hypertensive, beta-blocker, calcium channel blocker, and renin-angiotensin system antagonist drugs, number of comorbidities and number of concurrent drug treatments were significantly associated with the value of the ECG-derived parameters (**Table 20**). Age and gender affected all the parameters. EMS arrival time affected only AMSA and RMS, while the number of DF attempts had effects only for PF and MNF. As expected, beta-blocker treatment affected MDF and MNF. Comorbidities and treatments had different effects among the various parameters (**Table 20**).

More specifically, age, gender, EMS arrival, myocardial infarction, congestive heart failure, diabetes, number of comorbidities, and anti-hypertensive drug, were the factors associated with AMSA values.

Table 20. Relation between ECG parameters and baseline measurements. Univariate regression model.

Model		AMSA		RMS		PF		MDF		MNF	
		β	p value	β	p value	β	p value	β	p value	β	p value
Univariate	Age (years)	-0.0037	**<0.0001**	0.0005	0.6221	-0.0051	**<.0001**	-0.0042	**<.0001**	-0.0027	**<.0001**
	Gender (male)	0.1251	**<0.0001**	0.0866	**0.0051**	0.0788	**0.0014**	0.041	**0.0132**	0.0201	**0.0495**
	Arrival time (minutes)	-0.0075	**0.0168**	-0.0102	**0.0025**	0.0002	0.9482	0.0022	0.2162	0.0007	0.5088
	Number of defibrillations	-0.0056	0.4159	-0.0107	0.1484	0.0119	**0.0433**	0.0007	0.8608	0.0148	**<.0001**
	Myocardial infarction	0.252	**<0.0001**	0.2353	**<.0001**	0.0439	0.0621	0.0313	**0.0474**	-0.0074	0.4495
	Congestive heart failure	0.0749	**0.0094**	0.0865	**0.0053**	0.0109	0.6598	-0.0076	0.6479	-0.0269	**0.0086**
	Chronic Pulmonary Disease	0.0005	0.9902	-0.0425	0.3177	0.0529	0.1183	0.02	0.3803	0.0399	**0.0045**
	Diabetes	-0.151	**<0.0001**	-0.0298	0.4564	-0.1226	**0.0001**	-0.1171	**<.0001**	-0.0691	**<.0001**
	Renal disease	0.0137	0.7897	0.1097	**0.0468**	-0.0755	0.0858	-0.0955	**0.0012**	-0.0523	**0.0041**
	Number of comorbidities	0.0359	**0.0002**	0.0474	**<.0001**	-0.0013	0.8749	-0.0073	0.1809	-0.0139	**<.0001**
	Antithrombotic drugs	0.0413	0.1229	0.0872	**0.0024**	-0.0493	**0.0312**	-0.0423	**0.0059**	-0.0507	**<.0001**
	Cardiac therapy	0.0331	0.2849	0.0972	**0.0034**	-0.0585	**0.0271**	-0.0737	**<.0001**	-0.064	**<.0001**
	Antihypertensive drugs	0.268	**<0.0001**	0.2706	**<.0001**	0.1045	0.052	0.0854	**0.018**	-0.0186	0.4047
	Beta blocker drugs	-0.0253	0.3824	0.0129	0.6785	-0.037	0.1357	-0.0352	**0.0348**	-0.0275	**0.0075**
	Calcium channel blockers	-0.0507	0.0925	-0.0294	0.3645	-0.0426	0.0987	-0.0055	0.7519	-0.0089	0.4042
	Renin-angiotensin system antagonists	-0.0463	0.0692	-0.0791	**0.0039**	0.0128	0.5586	0.0317	**0.0304**	0.0071	**0.4328**
	Number of drugs	0.0241	0.1102	0.0381	**0.0187**	-0.0198	0.125	**-0.0151**	0.0819	-0.0197	**0.0002**

The analysis is adjusted for age and gender. The dependent variables are modeled as natural logarithm

Using a multivariate logistic regression model, including all variables individuated with the univariate analysis ($p<0.05$), factors that were predictors of DF success were individuated for each of the ECG-derived parameters (**Table 21**). More in details, factors to be considered for

AMSA were: age, gender, myocardial infarction, diabetes, anti-hypertensive drugs and calcium channel blocker agents (**Table 21**).

Table 21. Relation between ECG parameters and baseline measurements. Multivariate regression model

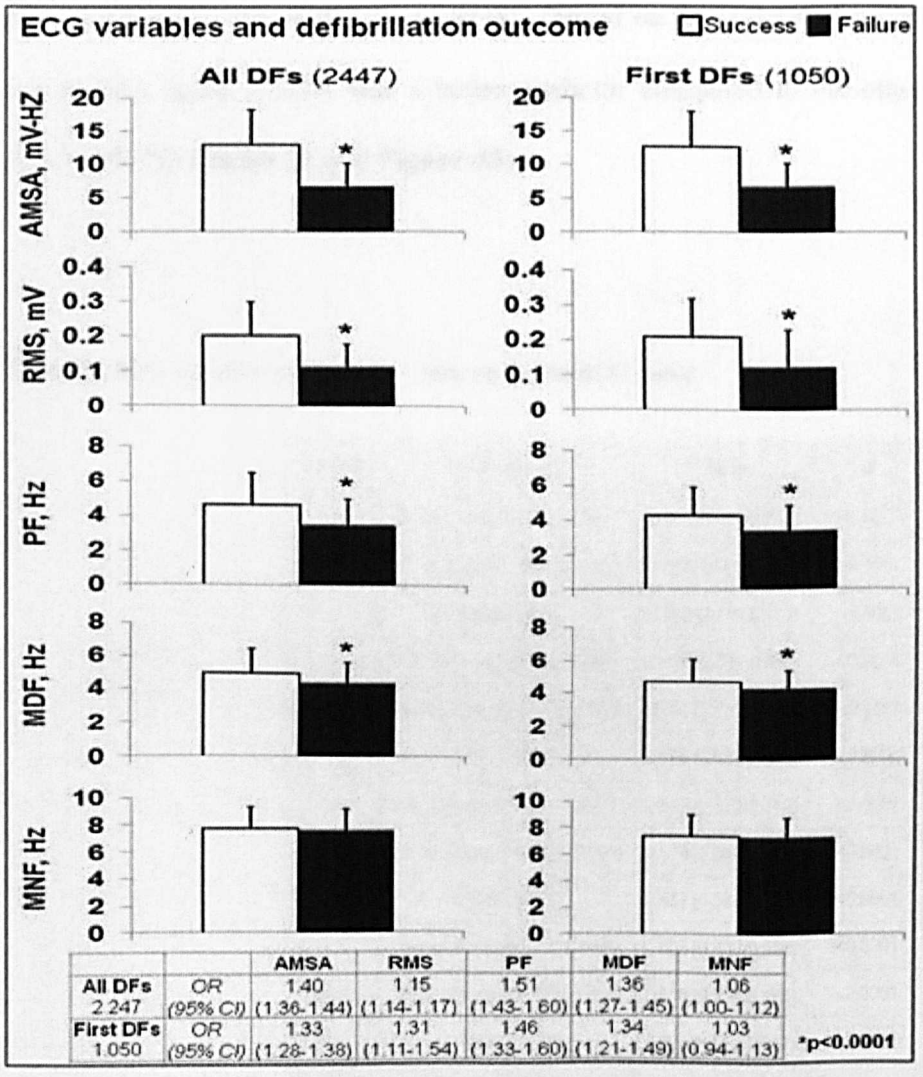
	Model	β	p value
Multivariate	**AMSA**		
	Age	-0.0032	0.0002
	Gender (male)	0.1298	<.0001
	Myocardial infarction	0.2482	<.0001
	Diabetes	-0.1639	<.0001
	Antihypertensive drugs	0.2468	<.0001
	Calcium channel blockers	-0.0689	0.0213
	RMS		
	Age	0.0000	0.9914
	Gender (male)	0.1139	0.0002
	Arrival time	-0.0091	0.0063
	Myocardial infarction	0.2177	<.0001
	Antihypertensive drugs	0.2364	0.0004
	Antithrombotic drugs	0.1262	<.0001
	Renin-angiotensin system antagonists	-0.1119	0.0001
	PF		
	Age	-0.0043	<.0001
	Gender (male)	0.0749	<.0001
	Myocardial infarction	0.0594	0.0132
	Chronic Pulmonary Disease	0.0817	0.0178
	Diabetes	-0.1219	0.0002
	Liver disease	-0.09859	0.0483
	Cardiac therapy	-0.0648	0.0186
	MDF		
	Age	-0.0032	<.0001
	Gender (male)	0.0343	0.0367
	Myocardial infarction	0.0541	0.0009
	Congestive heart failure	0.0429	0.0248
	Diabetes	-0.1227	<.0001
	Renal disease	-0.0753	0.0135
	Cancer	0.07042	0.0092
	Antithrombotic drugs	-0.0358	0.044
	Cardiac therapy	-0.0767	0.0002
	Beta blocker drugs	-0.0414	0.0201
	Renin-angiotensin system antagonists	0.0683	<.0001
	MNF		
	Age	-0.0019	<.0001
	Gender (male)	0.0167	0.0971
	Defibrillation attempts	0.0125	<.0001
	Chronic pulmonary disease	0.0549	<.0001
	Diabetes	-0.0652	<.0001
	Antithrombotic drugs	-0.0343	0.0012
	Cardiac therapy	-0.0505	<.0001
	Renin-angiotensin system antagonists	0.0273	0.0039

Cardiac therapy include: cardiac glycosides, anti-arrhythmics, inotropes, vasodilators.
The analysis is adjusted for age and gender. The dependent variables are modeled as natural logarithm

AMSA and other ECG-derived parameters and DF success

Among the 2.447 DF attempts, 26.2% were successful, while considering only the first DF attempts (1.050), 26.5% were successful. AMSA was significantly higher prior to a successful

DF than a failing one. Considering all DF attempts, AMSA values were 13 ± 5.2 and 6.8 ± 3.5 for successful and unsuccessful DFs respectively ($p<0.0001$, **Figure 44**). Considering only the first DF attempts, AMSA values were 12.6 ± 5.5 and 6.7 ± 3.6 for successful and unsuccessful DFs respectively ($p<0.0001$). All the other ECG-derived parameters, with the exception of MNF, were also significantly higher prior to a successful DF compared to an unsuccessful one (**Figure 44**).



		AMSA	RMS	PF	MDF	MNF
All DFs 2.247	OR (95% CI)	1.40 (1.36-1.44)	1.15 (1.14-1.17)	1.51 (1.43-1.60)	1.36 (1.27-1.45)	1.06 (1.00-1.12)
First DFs 1.050	OR (95% CI)	1.33 (1.28-1.38)	1.31 (1.11-1.54)	1.46 (1.33-1.60)	1.34 (1.21-1.49)	1.03 (0.94-1.13)

Figure 44. AMSA, RMS, PF, MDF, and MNF values for successful and not successful DF for all, and first attempts. In the bottom table: OR of the different ECG-derived parameters for all and first DF attempts.

Moreover, all the ECG-derived parameters, with the exception MNF, were independent predictors of DF success (ORs are reported in the bottom table in **Figure 44**). More

specifically, AMSA presented an OR of 1.40 (95% CI 1.36-1.44, p<0.01) for all DFs, and 1.33 (95% CI 1.28-1.38, p<0.01) for only the first DF attempts (**Figure 44**).

The area under the ROC curve for the prediction of DF success based on ECG-derived parameters are reported in **Table 22**. AMSA had the AUC significantly greater compared to all the other parameters and thereby was a better DF success predictor. More specifically AUCs for AMSA were 0.861 and 0.834 for all and first DF attempts (**Table 22** and **Figure 45**). Considering the prediction of ROSC (based on ECG-derived parameters measured prior to last DF), again AMSA was a better predictor compared to the other parameters, with an AUC of 0.753 (**Table 22** and **Figure 43**).

Table 22. ECG variables predictivity - Area under the ROC curve

Variable	Outcome (N)	AUC	p
AMSA	DF success (All DFs, 2.447)	0.861 (0.845-0.877)	<0.0001
	DF success (First DFs, 1.050)	0.834 (0.806-0.861)	<0.0001
	ROSC (860)	0.753 (0.719-0.787)	<0.0001
RMS	DF success (All DFs, 2.447)	0.810 (0.793-0.830)	<0.0001
	DF success (First DFs, 1.050)	0.795 (0.765-0.824)	<0.0001
	ROSC (860)	0.705 (0.668-0.740)	<0.0001
PF	DF success (All DFs, 2.447)	0.695 (0.672-0.719)	<0.0001
	DF success (First DFs, 1.050)	0.676 (0.640-0.712)	<0.0001
	ROSC (860)	0.637 (0.598-0.675)	<0.0001
MDF	DF success (All DFs, 2.447)	0.625 (0.600-0.650)	<0.0001
	DF success (First DFs, 1.050)	0.616 (0.578-0.654)	<0.0001
	ROSC (860)	0.596 (0.558-0.637)	<0.0001
MNF	DF success (All DFs, 2.447)	0.523 (0.500-0.550)	0.0825
	DF success (First DFs, 1.050)	0.505 (0.465-0.544)	0.8139
	ROSC (860)	0.511 (0.470-0.551)	0.7661

Comparison between different AUCs are significantly different

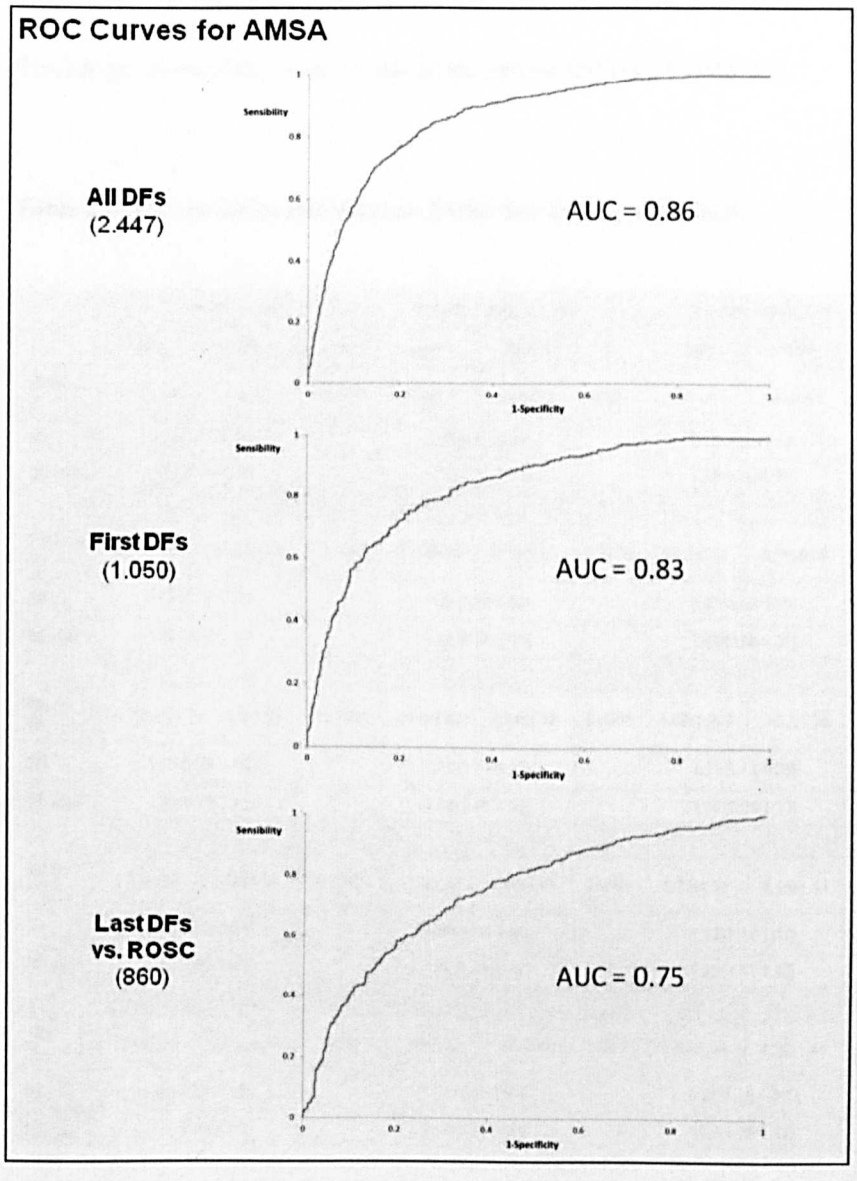


Figure 45. ROC curves for AMSA and DF outcome prediction for all and first DFs, and for AMSA (last DFs) and ROSC.

AMSA and other ECG-derived parameters and outcome

Relationship between ECG-derived parameters and ROSC and long-term outcomes were also assessed (**Table 21**). All the ECG-derived parameters, with the exception of MNF, were independently associated with ROSC (RRs are reported in **Table 23**). More specifically,

AMSA presented an adjusted OR for ROSC prediction of 1.21 (95% CI 1.16-1.25, per mV-Hz increase, p<0.01, **Table 23**). Surprisingly, AMSA and all the other ECG-derived parameters, with the exception of RMS, were also independent predictors for survival at hospital discharge, 6-months, and 1 year after resuscitation (**Table 23**).

Table 23. ECG variables and outcome: ROSC and long term survival.

	ROSC (N=860)			Hospital discharge^ (N=308)			6-month survival^ (N=308)			1-year survival^ (N=308)		
	yes	no	p	alive	dead	p	alive	dead	p	alive	dead	p
AMSA, mV-Hz	11.7±5.3	7.4±4.1	<0.0001	12.6±5.3	10.8±5.1	0.0017	13.1±5.3	10.6±5.1	<0.0001	13.1±5.1	10.8±5.3	<0.0001
RR	1.22 (1.17-1.26)			1.05 (1.02-1.09)			1.06 (1.03-1.10)			1.05 (1.02-1.09)		
RR adj.*	1.21 (1.16-1.25)			1.06 (1.02-1.10)			1.05 (1.02-1.09)			1.05 (1.02-1.09)		
RMS, mV	0.18±0.10	0.12±0.075	<0.0001	0.18±0.09	0.18±0.12	0.2378	0.18±0.12	0.18±0.08	0.1269	0.18±0.08	0.18±0.12	0.1942
RR	1.09 (1.07-1.11)			1.00 (0.98-1.02)			1.00 (0.98-1.02)			1.00 (0.98-1.02)		
RR adj.*	1.09 (1.07-1.12)			1.00 (0.98-1.01)			1.00 (0.98-1.01)			1.00 (0.98-1.01)		
PF, Hz	4.35±1.81	3.52±1.50	<0.0001	4.74±1.82	3.94±1.71	0.0001	4.89±1.842	3.91±1.66	<0.0001	4.95±1.83	3.95±1.68	<0.0001
RR	1.36 (1.24-1.49)			1.20 (1.09-1.33)			1.23 (1.12-1.35)			1.23 (1.12-1.35)		
RR adj.*	1.31 (1.18-1.45)			1.18 (1.05-1.30)			1.20 (1.09-1.33)			1.19 (1.09-1.32)		
MDF, Hz	4.83±1.51	4.34±1.34	0.0003	5.14±1.47	4.48±1.49	0.0003	5.27±1.51	4.45±1.42	<0.0001	5.31±1.47	4.49±1.45	<0.0001
RR	1.27 (1.15-1.41)			1.28 (1.14-1.45)			1.32 (1.18-1.47)			1.29 (1.18-1.45)		
RR adj.*	1.21 (1.07-1.35)			1.22 (1.06-1.39)			1.25 (1.11-1.43)			1.23 (1.10-1.39)		
MNF, Hz	7.50±1.47	7.42±1.39	0.8556	7.74±1.39	7.23±1.48	0.0051	7.86±1.38	7.20±1.46	0.0002	7.87±1.34	7.24±1.49	0.0004
RR	1.04 (0.94-1.15)			1.19 (1.05-1.33)			1.23 (1.10-1.37)			1.22 (1.09-1.35)		
RR adj.*	1.00 (0.90-1.13)			1.19 (1.04-1.35)			1.20 (1.08-1.33)			1.19 (1.06-1.33)		

*Adjusted for age, gender and factors identified in the multivariate analysis

Threshold AMSA values for DF outcome prediction

All DF attempts - Using the intersection of sensitivity, specificity, and accuracy curves (**Figure 46**), an AMSA threshold of 8.9 mV-Hz, provided a balanced sensitivity, specificity

and accuracy of 79%, with a NPV of 91% and a PPV of 56%. An AMSA threshold of 15.5 mV-Hz, instead, provided a balanced accuracy and PPV of 79% in predicting DF success, with a specificity of 97%. Higher AMSA thresholds were associated with further increases in PPV and specificity, i.e. AMSA > 23 mV-HZ led to a PPV of 100% (**Figure 46**). For low AMSA thresholds, the majority of unsuccessful DFs were correctly predicted with high sensitivity and NPV (**Figure 46**). AMSA < 6.5 mV-Hz yielded a NPV > 95% (**Figure 46**). Lower thresholds further improved NPV.

First DF attempts - Using the intersection of sensitivity, specificity, and accuracy curves (**Figure 46**), an AMSA threshold of 8.6 mV-Hz, provided a balanced sensitivity, specificity and accuracy of 76%, with a NPV of 89% and a PPV of 54%. An AMSA threshold of 16 mV-Hz, instead, provided a balanced accuracy and PPV of 77% in predicting DF success, with a specificity of 97%. Higher AMSA thresholds were associated with further increases in PPV and specificity, i.e. AMSA > 23 mV-HZ led to a PPV of 100% (**Figure 46**). For low AMSA thresholds, the majority of unsuccessful DFs were correctly predicted with high sensitivity and NPV (**Figure 46**). AMSA < 5 mV-Hz yielded a NPV > 95% (**Figure 46**). Lower thresholds further improved NPV.

Last DF attempts and ROSC - Using the intersection of sensitivity, specificity, and accuracy curves (**Figure 46**), an AMSA threshold of 8.7 mV-Hz, provided a balanced sensitivity, specificity and accuracy of 70%, with a NPV of 80% and a PPV of 56%. An AMSA threshold of 13.2 mV-Hz, instead, provided a balanced accuracy and PPV of 72% in predicting ROSC, with a specificity of 92%. Higher AMSA thresholds were associated with further increases in PPV and specificity, i.e. AMSA > 26 mV-HZ led to a PPV of 100% (**Figure 46**). For low AMSA thresholds, the majority of unsuccessful DFs were correctly predicted with high

sensitivity and NPV (**Figure 46**). AMSA < 3 mV-Hz yielded a NPV > 90% (**Figure 46**). Lower thresholds further improved NPV.

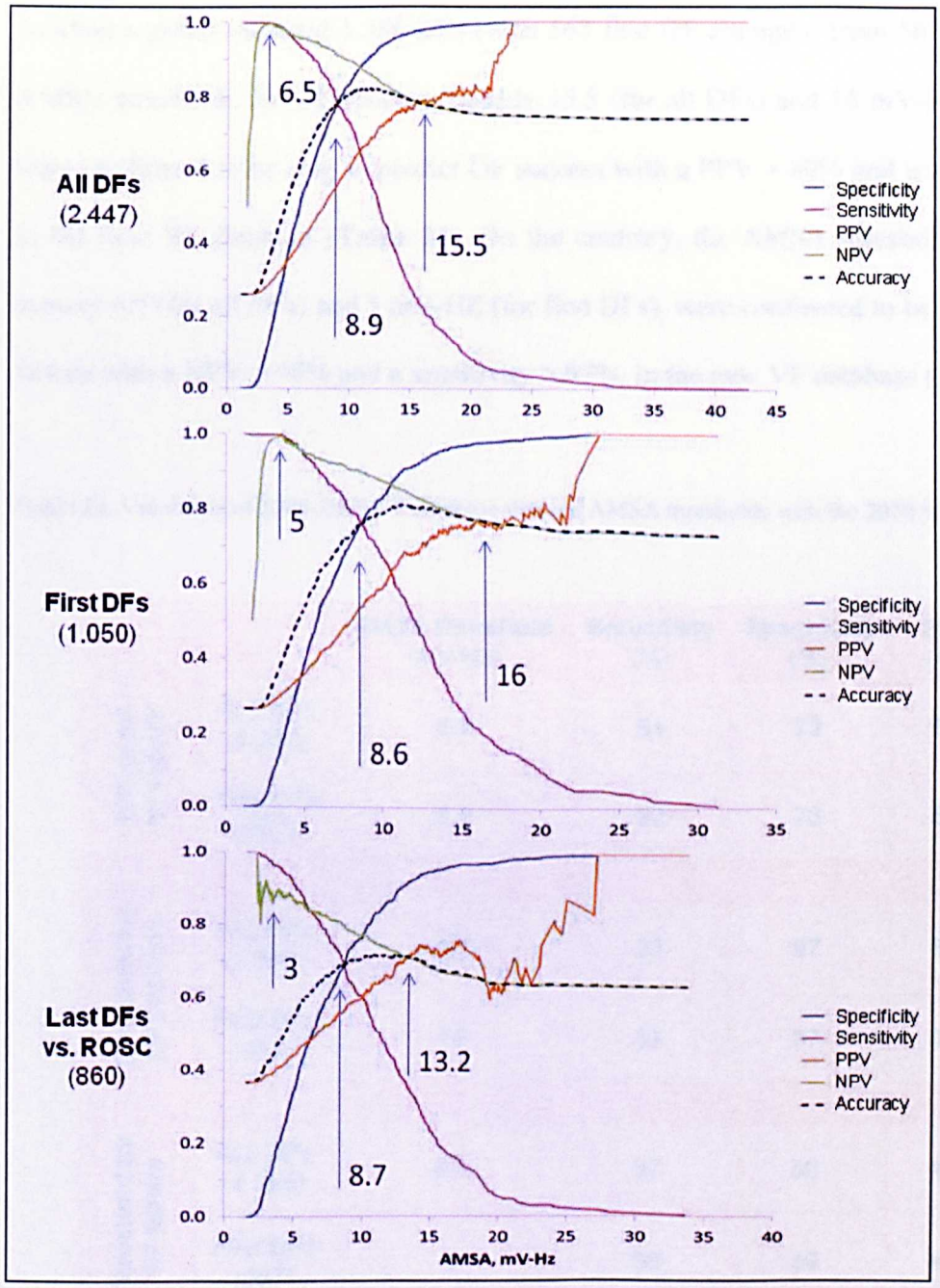


Figure 46. Sensitivity, specificity, accuracy, and positive and negative predictive value (PPV and NPV) curves for different AMSA values, in all DFs, first DFs, and last DFs.

Validation of AMSA thresholds

AMSA thresholds for successful and unsuccessful DFs (all DFs and first DFs) defined in the derivation group (**Figure 46**) were then validated in the validation group (**Table 24**). The validation group included 1.386 DFs (with 567 first DF attempts) from 567 VF patients. The AMSA thresholds for DF success, namely 15.5 (for all DFs) and 16 mV-HZ (for first DFs), were confirmed to be able to predict DF success with a PPV > 80% and a specificity of 97%, in the new VF database (**Table 24**). On the contrary, the AMSA thresholds for DF failure, namely 6.5 (for all DFs) and 5 mV-HZ (for first DFs), were confirmed to be able to predict DF failure with a NPV > 98% and a sensitivity > 97%, in the new VF database (**Table 24**).

Table 24. Validation of 2008-2009 VF database-derived AMSA thresholds with the 2010 VF database

		AMSA threshold (mV-Hz)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Balanced threshold	*ALL DFs (1.386)*	8.9	91	72	60	95
	First DFs (567)	8.6	92	73	60	95
Threshold for DF success	*ALL DFs (1.386)*	15.5	35	97	82	76
	First DFs (567)	16	33	97	80	77
Threshold for DF failure	*ALL DFs (1.386)*	6.5	97	50	47	98
	First DFs (567)	5	99	39	41	99

DISCUSSION

The ***main findings*** of our research can be summarized as follows:

1. Identification of a specific pathway, the kynurenine pathway, that is activated early in the post-resuscitation period and that is associated with the severity of post-cardiac arrest syndrome. Indeed, we have demonstrated that KP is activated in rats, pigs, and humans, after ROSC and this activation persists during the initial days post-cardiac arrest. Moreover, in a large cohort of cardiac arrest patients, we have furher demonstrated that ICU admission levels of KP metabolites were associated with the duration of cardiac arrest, i.e. time to ROSC, and with the severity of post-cardiac arrest shock during the first 24 hours. In addition, KP metabolites predicted ICU mortality and 12 month outcome. KP therefore may play a pathophysiological role in the severity of shock, early death and poor neurological outcome after resuscitation. KP metabolites, and especially KYNA and 3-HAA, may have a clinical utility for cardiac arrest outcome prognostication.

2. Confirmation and validation, in two large databases (one from US and one from Italy) of out-of-hospital VFs, that AMSA is one of the best ECG-derived parameters to be used as predictor of defibrillation outcome. Indeed, AMSA was capable of predicting DF outcome with high accuracy. AMSA was significantly higher prior to a successful DF than prior to an unsuccessful one. Thresholds for prediction of successful and unsuccessful DFs were similar in both databases, i.e. 16-17 mV-Hz for success and < 7 mV-Hz for failure. AMSA also showed additional promise for monitoring the effectiveness of chest compression. Finally, for the first time, it has been showed association between AMSA and different factors, such as age, gender, EMS arrival, heart disease, diabetes and cardiovascular drugs.

The Discussion is organized based on the two main topics of the researches described in the previous pages: *kynurenine pathway* and *amplitude spectrum area.*

Kynurenine pathway

Emerging metabolome profiling technologies offer the possibility of identifying novel biomarkers and pathways activated in cardiovascular diseases; however, applications to post-resuscitation myocardial dysfunction in the setting of cardiac arrest are still lacking. We used a global LC-MS metabolomic approach to obtain a comprehensive view of changes in plasma metabolites associated with CPR in an established and widely accepted rat model. The application of metabolomic analysis in cardiovascular diseases is an emerging field and it is not yet possible to depict any single metabolic picture responsible for the prediction and progression of cardiovascular disease. However, the identification of clinically relevant changes in circulating metabolites is opening exciting avenues in the cardiovascular field *(Alexander 2001, Barderas 2011, Lewis 2008, Mayr 2008 and 2009)*. For example, Shah and colleagues *(Shah 2010)* showed that a signature composed of dicarboxyacylcarnitines was predictive of further cardiovascular events in patients with coronary artery disease. An important role of phospholipids as new key culprits in atherosclerosis was highlighted in patients with cardiovascular disease and a pathological role of ketone bodies has been suggested for human atrial fibrillation *(Wang 2008)*. In acute ischemia and acute myocardial diseases many intermediates of the citric acid cycle were found to be depressed as a direct consequence of myocardial ischemia *(Sabatine 2005, Zhao 2008)*.

Hence, we first adopted an unbiased strategy towards profiling as many plasma metabolites as possible in the very early post-resuscitation phase (2 hr post-ROSC) in rats. The profiling data was then used to detect altered biochemical processes using bioinformatics-based pathway mapping. Indeed, statistically significant signatures could be obtained in rats' plasma in the very early CPR period compared to control rats. Many biochemical alterations were in line with those already reported in the literature about cardiovascular dysfunctions, supporting the

feasibility and robustness of our explorative untargeted LC-MS strategy. For example, there were changes in the plasma levels of some saturated and unsaturated fatty acids, with an overall tendency to decrease in the early CPR phase. This de-regulation may have significant energetic and functional consequences on the heart, affecting the delivery of free fatty acids to the myocardium and their utilization during ischemia and following reperfusion. The involvement of fatty acid beta-oxidation in rats in the early CPR phase is consistent with the decrease in the myocardial capacity for this process in rodent models of heart failure *(Lopaschuk 2010)* and in clinical settings *(Jaswal 2011, Neglia 2007)*. Our findings are in accordance with a general reduction in oxidative fuel metabolism and greater reliance on anaerobic metabolism for energy revealed by metabolomic analysis in clinical settings of myocardial ischemia/reperfusion *(Turer 2009)*.

There is convincing body of the role of sphingolipids in cardiovascular dysfunction and in particular for S1P, which has potent effects against acute ischemia-reperfusion injury *(Baranowski 2011, Knapp 2011)*. In line with these observations, in rat plasma from the early CPR phase, we found a reduction in the level of S1P, which could reduce endogenous protective action on cardiomyocyte viability *(Knapp 2009)*.

Analysis of rat plasma using a comprehensive LC-MS platform also identified metabolites of the essential aminoacid TRP catabolism that had not, to our knowledge, been previously associated with any post-resuscitation syndrome. We identified what we believe to be novel changes in plasma metabolites after CPR. The targeted LC-MRM-MS platform with stable-isotope dilution method enabled us to unambiguously quantify the statistically meaningful changes of TRP and KP metabolites throughout the post-resuscitation observational period. Methodological refinements, including optimization of MS conditions, and deuterated standards resulted in a sensitive, selective and accurate method for the simultaneous

measurements of TRP, KYN, KYNA, and 3-HAA in plasma. Baseline values were similar to those reported in literature *(Fukushima 2009, Midttun 2009, Pawlak 2001, Zheng 2012)*.

The subsequent study with targeted metabolomics confirmed that KP was activated following resuscitation from cardiac arrest in three different species. Accordingly, KP activation was initially observed in resuscitated rats, was then validated in pigs subjected to cardiac arrest and CPR, and was ultimately confirmed in a small cohort of human patients. Indeed, increases in plasma levels of KP metabolites, KYN, KYNA and 3-HAA, occurred during the initial hours following resuscitation and persisted up to 3 to 5 days following cardiac arrest. KP activation showed an equivalent time course in rats, pigs, and humans, and was significantly related to the severity of post-cardiac arrest myocardial dysfunction, cerebral injury, functional outcome and survival.

KP is a major pathway of the catabolism of the essential aminoacid TRP. Specifically, two enzymes initiate the KP: tryptophan 2,3-dioxygenase (TDO), that is mainly present in the liver and is stimulated by glucocorticoids; and indoleamine 2,3-dioxygenase (IDO), that is widely expressed in a variety of human tissues, such as the brain, kidney, lung, spleen, and duodenum, as well as in macrophages and dendritic cells and is stimulated by proinflammatory cytokines, including interferon-γ, TNFa, IL-1 and 2, and by lipopolysaccharides and free radicals *(Maes 2011, Wilson 2012)*. Indeed, upon inflammatory stimulation, IDO is induced and consequently KP activated *(Changsirivathanathamrong 2011, Wilson 2012)*. Similarly to a sepsis-like syndrome, a systemic inflammatory and immune response is observed after CPR, and might be the trigger for IDO induction *(Adrie 2004, Nolan 2008, Peberdy 2010, Stoppe 2012)*. In the present study, early KP activation has been consistently observed in both small and large animals resuscitated from cardiac arrest, and ultimately

confirmed in humans. In our study, systemic inflammation was present in the 5 resuscitated patients, as demonstrated by circulating levels of TNFa, IL-8, MIF, and CRP, while association between KP activation and myocardial oxydative stress, i.e. isoprostanes, has been observed in the rat model.

Altered TRP metabolism has been described as a hallmark of many stress related situations *(Clarke 2009, Maes 2011)*. In these settings of glucocorticoid overdrive, in fact, TDO is activated together with a super induction of IDO in response to stress *(Maes 2011)*. Concurrently elevated circulating catecholamines have been reported to contribute to reduction in total TRP concentrations. Thus, rodents subjected to stressful conditions, i.e. forced swimming or immobilization, a low TRP diet increased adrenal weight, plasma corticosterone levels and reactivity to stimuli; administration of TRP, instead, had acute antianxiety-like effects *(Maes 2011, Wong 2001)*. Indeed, cardiac arrest is characterized by increases in plasma cortisol level and catecholamine release. A trend toward lower serum cortisol level in survivors than in non survivors has been earlier reported *(Hékimian 2004, Maes 2011)*. The stress of whole body ischemia/reperfusion that follows cardiac arrest might be therefore another component affecting the described KP activation after CPR.

It has been proposed that TRP catabolism through the KP may contribute to oxidative stress and brain damage following ischemia *(Darlington 2007)*. Contributors to the development of ischemia-induced cerebral injury are, in fact, post-ischemia neuroimmune and inflammatory reactions *(Neumar 2000, Nolan 2008)*. Accordingly, one component of this network is the KP *(Darlington 2007, Stone 1993)*. Indeed, KYN is the first KP metabolite, that is further metabolized to the neurotoxic 3-HAA and its derivatives, quinolinic acid and picolinic acid (QA and PA). 3-HAA exerts its neurotoxic actions by inducing both cerebral oxidative stress and

excitotoxicity through activation of N-Methyl-D-Aspartate (NMDA)-receptors by QA and PA. 3-HAA's metabolites have not only neuroexcitatory effects, but also neurotoxic ones, causing neuronal, astrocyte and microglial cell injury, destruction of postsynaptic elements, and reductions in cerebral cholinergic circuits *(Brouns 2010, Darlington 2007, Sas 2007, Stone 1993)*. KYNA is another KYN metabolite, produced by astrocytes, in response to increased KYN level. KYNA has neuroprotective properties related to its activity as NMDA antagonist *(Nozaki 1992, Sas 2007, Stone 2002)*. KYNA generation might represent an adaptive response in order to block the potentially harmful effects of excessive glutamate receptor stimulation that follows an ischemic insult *(Neumar 2000)*. The correlations between post-resuscitation 3-HAA plasma levels and cortical and hippocampal histological lesions and neurological deficit scores in pigs, suggest a possible role of KP metabolites in post-resuscitation cerebral injury and neurological dysfunction also in the instance of cardiac arrest. Together with increases in plasma 3-HAA, there were concurrent increases in plasma KYNA and these were consistently seen in the 3 species.

In support of our results, KP activation, expressed as KYN to TRP ratio, has been shown to correlate with stroke severity and long-term outcome in stroke patients *(Brouns 2010, Darlington 2007)*. Patients with poor outcome had higher KYN/TRP ratio than patients with more favorable outcome. Moreover, experimental blockade of the KP has been reported to reduce cerebral infarct volume in a model of cerebral ischemia and reperfusion *(Cozzi 1999)*.

It has been reported that in response to generation of KP's neuroprotective metabolite KYNA, microglia and macrophages may produce even greater amounts of neurotoxic KP metabolites, i.e. 3-HAA *(Sas 2007, Wilson 2012)*. This loop of events may explain TRP and KP metabolites levels observed in our animals subjected to hypothermia. Therapeutic hypothermia is a well

established protective intervention against brain ischemia/reperfusion injury after cardiac arrest *(Peberdy 2010, Polderman 2009)*. TH, in fact, suppresses many of the reactions associated with reperfusion injury, i.e., inflammatory responses and intracellular injury pathways, including activation of pro-apoptotic enzymes. Indeed, our pigs treated with TH presented better neurological recoveries and lesser cerebral lesions compared to those that did not receive TH. These hypothermia-treated animals also presented lesser TRP catabolism compared to normothermic ones. The higher TRP plasma levels in these animals were accompanied by higher levels of neuroprotective KYNA. However, hypothermic animals also presented significant increases in plasma concentrations of neurotoxic 3-HAA, as an hypothesized counteracting response to neuroprotection provided by TH *(Nolan 2008, Peberdy 2010, Polderman 2009)*. Nevertheless, a specific effect of TH on KP activation has to be considered with caution. The levels of KP metabolites were, in fact, assessed at the 5th day after cardiac arrest. Any direct and early effect played by TH on KP could not be investigated due to the lack of an earlier blood sampling, as a limitation of the retrospective study design.

In further support of potential implications of KP in post-resuscitation brain injury and outcome and in neuroprotective effects played by KYNA, are the additional investigation of KP in pigs treated with a well-known neuroprotective intervention, namely inhalation of the noble gas xenon. Although noble gases are traditionally believed to be 'inert', these monoatomic colorless and odorless agents are indeed capable to interact with amino-acids in the active sites of several enzymes and receptors, producing biological effects *(Jawad 2009)*. Especially, xenon has been widely explored in several neurological injury models, including cardiac arrest and clinical phase II trials are currently underway to prove its benefits *(Fries 2008 and 2012)*. Indeed, in our pigs, administration of xenon translated to improved early neurologic and neurocognitive recovery and this was accompanied by no changes in post-resuscitation

plasma levels of TRP and 3-HAA, and by a significant increase in post-resuscitation plasma levels of the neuroprotective metabolite KYNA. This increase in KYNA after xenon treatment may be related to a concurrent action as NMDA antagonist played by xenon itself, which may lead to KYNA preservation.

More importantly, KP activation has been associated not only with brain injury but it may also concur to the pathogenesis of systemic inflammatory response syndrome and sepsis *(Changsirivathanathamrong 2011, Huttunen 2010, Jung 2009, Sas 2007, Wang 2010)*. IDO activity was markedly increased in 132 patients with bacteremia, in whom KP was significantly more activated in non-survivors compared to survivors *(Huttunen 2010)*. In 60 major trauma patients, significantly increased KYN was detectable already within 24 hr after hospital admission in blood from patients who later developed sepsis *(Logters 2009)*. In those patients KP activation predicted subsequent sepsis development, multiple organ failure, and survival. Most likely early post-traumatic inflammation in conjunction with augmented circulating pro-inflammatory cytokines and neutrophil activation resulted in IDO stimulation and consequent KP activation. The above events may occur also in cardiac arrest patients *(Adrie 2002 and 2004, Nolan 2008, Peberdy 2010)*. Indeed, activation of the inflammatory cytokine cascade, chemokine upregulation and ultimately recruitment of inflammatory leukocytes and reactive astrogliosis have been reported after cardiac arrest and play major roles in the final outcome. From this point of view, the reported anti-inflammatory effect of KYNA, through the inhibition of TNFa, may provide an interesting feed-back mechanism in this post-cardiac arrest inflammatory response *(Wang 2006)*.

KP also concurs to the pathogenesis of vasoplegia and hypotension during septic shock *(Changsirivathanathamrong 2011, Jung 2009, Wang 2010)*. IDO expression was, in fact, induced in the

endothelial cells of small resistance vessels and contributed to the dysregulation of vascular tone during systemic inflammation due to endotoxemia in mice *(Wang 2010)*. This increased IDO activity accounted for a greater production of KYN, that directly mediated arterial relaxation through soluble guanylate cyclase activation *(Changsirivathanathamrong 2011)*. A clear protection against hypotension due to septic shock and a reduced mortality has been reported in IDO knockout mice or in mice treated with a specific IDO inhibitor, 1-methyl-D-tryptophan *(Jung 2009)*. Those mice also presented decreased levels of the pro-inflammatory cytokines, TNFa, IL-6, and IL-12, and enhanced levels of the anti-inflammatory IL-10. A recent clinical study confirmed the above experimental results on 16 septic shock patients, in which IDO activity increased up to 9-fold *(Changsirivathanathamrong 2011)*. Moreover, inotrope requirements were strongly correlated with IDO activity in those septic patients *(Changsirivathanathamrong 2011)*.

The above events may occur also in the setting of cardiac arrest *(Nolan 2010, Peberdy 2010)*. As shown in our study, post-cardiac arrest myocardial dysfunction, with low ejection fraction, cardiac output, stroke volume, together with profound arterial hypotension, were consistently reported in rats and pigs. More importantly, in our rat model, plasma levels of KP metabolites, KYNA and 3-HAA, increased concurrently to decreases in myocardial function and increases in biomarkers of heart injury and oxidative stress. Similarly, the severity of post-resuscitation arterial hypotension and reduction in cardiac output were significantly related to increases in KYNA and 3-HAA in pigs.

The subsequent clinical study confirmed in a large cohort of patients that the TRP catabolism through the KP is activated early after resuscitation from cardiac arrest. Patients who died before ICU discharge exhibited significantly higher levels of KP metabolites at ICU admission

suggesting a higher degree of KP activation and KP metabolites levels predicted early outcome after resuscitation. More importantly, KP metabolites, KYNA and 3-HAA, were associated with long term outcome.

Similarly to a sepsis-like syndrome, a systemic inflammatory and immune response is observed after CPR and the magnitude of the inflammatory response correlates with outcome as well as the severity of circulatory shock in cardiac arrest patients *(Adrie 2002)*. The inflammatory response commonly seen after cardiac arrest seems to be related to the duration of ischemia *(Adrie 2002)*. Adrie and colleagues *(Adrie 2002)* showed that after cardiac arrest an inflammatory response is seen and its magnitude is similar to that of severe sepsis. In their study, however, levels of various cytokines were not independent predictors of survival but this may have been due to low study power. The KYN/TRP ratio, which is regarded as an indicator of IDO activity, has been shown to predict case fatality and severity of shock in bacteremic patients *(Huttunen 2010)*. Huttunen et al. reported similar levels of TRP but higher levels of KYN and KYN/TRP in patients with fatal bacteremia *(Huttunen 2010)*. In our study, patients exhibiting a high KP activation at hospital admission subsequently had a more severe shock and were more likely to die in the ICU.

KP can be activated in the periphery as well as in the nervous system. Peripheral KYN readily crosses the BBB and is taken up by glial cells, while KYNA, 3-HAA and QA do not *(Fukui 1991, Speciale 1990)*. In the central nervous system further metabolism is segregated and under physiologic conditions, after the influx of KYN into the brain equal amounts of KYNA and 3-HAA are produced *(Guidetti 1995)*. Indeed, KYN concentration within the central nervous system is controlled by three main mechanisms: KYN movement from the peripheral circulation, where peripheral IDO and TDO are induced, across the BBB through the large

neutral amino acids transporter *(Stone 1993)*; basolateral secretion of KYN from BBB cells such as endothelial cells and pericytes *(Owe-Young 2008)*; and KYN synthesis from TRP within the central nervous system by astrocytes and microglia *(Fukui 1991, Maes 2011, Owe-Young 2008, Wilson 2012)*. However, in the instance of cardiac arrest, post-resuscitation inflammatory and immune system activation, might activate IDO both systemically and in the brain, and a larger amount KP metabolites may cross BBB in regions where it has been made more permeable by local ischemia-induced damage *(Brouns 2010, Darlington 2007)*. As discussed above, the balance of downstream KYN's metabolites, KYNA and 3-HAA, will modulate the resulting neurological injury *(Darlington 2007)*. Interestingly, also in our larger clinical study, both KYNA and 3-HAA increased similarly after cardiac arrest to further remark the complex balance and relationship between the generation of neuroprotective and neurotoxic KP metabolites after an ischemic insult. More importantly, the KYN/TRP ratio was higher in those with a poor outcome at 12 months and the absolute levels were comparable to those seen in stroke patients by Brouns and Darlington in stroke events, i.e. between 0.04 and 0.09 *(Brouns 2010, Darlington 2007)* Both KYNA and 3-HAA were independent predictors of long-term outcome after resuscitation from cardiac arrest.

Our large clinical study also confirmed the relationship between KP activation and the severity of post-cardiac arrest shock, previously observed in the animals. Indeed, higher levels of KYN, KYNA and KYN/TRP ratio were significantly associated with lower systolic blood pressure. Nevertheless, the relationship between KP and blood pressure seems to be more complex in the setting of cardiac arrest. In sepsis, in fact, high KYN/TRP activity was associated with high vasopressor requirements *(Darlington 2007)*. Absolute levels of KYN/TRP in our study were comparable to septic patients *(Changsirivathanathamrong 2011)* with a fairly low requirement of inotropes. Inotrope requirement during post-cardiac arrest is in general fairly

low and high requirement correlates with poor outcome *(Laurent 2002)*. In stroke patients on the other hand it is intriguing that despite high activation of KP, hypotension is uncommon *(Brott 1998)*. Thus, this finding contrasts the hypothesis that activation of the KP per se causes vasodilatory shock and hypotension. Clearly the interplay between shock, neurological damage and systemic inflammation seems to be complex. Further studies should look at where organ specific activation of the KP occurs, especially measured in the cerebrospinal fluid.

We recognize limitations in the interpretations of our findings. First, the experimental design was purely observational and focused on demonstration of post cardiac arrest activation of KP, thus it did not allow to identify causal relations: changes in circulating molecules may be the consequence as well as the cause of the observed pathophysiologic alterations. In order to investigate direct effects of KP activation on outcome of cardiac arrest, experiments that include administration of specific IDO inhibitors are planned. Nevertheless, this is the first evidence of consistent KP activation after cardiac arrest in animals and humans. Second, the studies were performed in healthy animals and therefore effects of underlying coronary disease in KP activation remain to be investigated. Third, animals were anesthetized and general anesthesia might have influenced stress response and KP activation *(Wilson 2012)*. However, the consistency of the results further strengthens our findings. Furthermore, results obtained from the 155 patients resuscitated from cardiac arrest provide exhaustive clinical confirmation on activation of KP after resuscitation and its implication with the severity of post–cardiac arrest syndrome and outcome. On the contrary, the strengths of the clinical study include: 1. the sample size which makes it the largest biohumoral study in out-of-hospital cardiac arrest to date; 2. robustness of clinical data; 3. representativeness of the population; and 4. outcome determined prospectively by a qualified independent assessor.

Amplitude spectrum area

Current DF algorithms are static in the sense that they do not consider the passage of time and the pathophysiology of the arrested myocardium *(Deakin 2010, Link 2010, Weisfeldt 2002)*. In our setting, the standard DF approach led to a successful DF in only 25-26% of attempts. The ability of AMSA to predict DF success is therefore of great importance, since this may allow to optimize timing of DF delivery *(Callaway 2005)*. There is also a potential to reduce CC interruptions and minimize myocardial damage by limiting repetitive and unnecessary DFs *(Cheskes 2011, Snyder 2004, Steen 2003, Yu 2002, Xie 1997)*.

There is evidence that VF changes over time and that it is possible to predict the success of a DF attempt by analysing the VF waveform features *(Callaway 2005, Eftestøl 2004, Endoh 2011, Li 2008b, Link 2010, Pernat 2001, Povoas 2002, Ristagno 2008b, Shanmugasundaram 2012, Sun 2011, Young 2004)*. Our studies are the first that investigated the ability of AMSA to predict DF success in large databases of out-of-hospital VFs, from US (609 patients) and Italy (1.617 patients). Beside the large number of patients enrolled, the studies have several additional strengths: 1. different defibrillators (ZOLL, Philips, and PhysioControl) have been used and thereby AMSA was tested with different DF waveforms (biphasic truncated exponential (BTE) and rectilinear biphasic (RLB)), with different energy delivery algorithms (no lower than 150 J for BTE and no lower than 120 J for RLB), and different trans-thoracic impedance compensation techniques (time-based compensation or current-based compensation) *(Kette 2013)*; 2. AMSA was investigated as predictor for both short and long term outcome, to test a potentially new use of AMSA as a prognosticator in cardiac arrest; 3. several factors, i.e. time of EMS arrival, co-morbidities and drug treatment were known in the study conducted in Italy and their impact on AMSA and other ECG-derived parameters was evaluated. Accordingly, the US study had as main endpoint to individuate AMSA thresholds for DF outcome and to investigate

relationships between AMSA and CC depth and between AMSA and resistant and recurrent VFs. The Italian study, beside individuating and confirming AMSA thresholds in a different population, through a consecutive derivation/validation approach, was also focused on investigating relationships between AMSA and ROSC and survival, and with potential affecting factors, described above. These studies therefore provide a lot of information on the use of AMSA and represent a valid basis for design a prospective intervention with a real time AMSA analysis as a guide for CPR manoeuvres.

AMSA values and thresholds predictive of DF outcome were similar in the two different databases. Indeed, we have confirmed AMSA to be significantly higher prior to a successful DF than prior to a failing one. While an AMSA of approximately 9-10 mV-Hz was able to predict DF outcome with a balanced sensitivity, specificity and accuracy of approximately 80%, higher AMSA values increased accuracy, specificity and PPV for predicting DF success. An AMSA threshold of 16-17 mV-Hz, in fact, was associated with up to 80% DF success, which could potentially be used to guide towards earlier DF as the likelihood of success is high. AMSA values greater than 23 mV-Hz (which was found, however, in a minority of patients) correctly predicted the success of DF with a PPV value of 100%, in the Italian database.

Animal studies have consistently demonstrated accuracy of AMSA for predicting DF success, yielding a balanced sensitivity and specificity of approximately 90%, with a NPV of 95% and a PPV of 78% *(Pernat 2001, Povoas 2002)*. Moreover, utility of AMSA as predictor of ROSC has been reported independent of myocardial substrate, i.e. in a swine model of ischemic VF *(Indik 2011)*. Two small retrospective clinical studies, including 46 and 90 VF patients, subsequently confirmed the above sensitivity and specificity *(Ristagno 2008b, Young 2004)*. In the present

investigations, although AMSA was able to predict with satisfactory accuracy DF outcome, balanced sensitivities, specificities, and PPVs were lower compared to the earlier reports. We believe, however, that current results are more realistic due to the large databases employed, that were approximately 10 to 20-fold greater than earlier ones and represent the largest studied to now for VF waveform analysis. Indeed, a good DF predictor should be both sensible and specific. For this reason, a balanced sensitivity and specificity of 80%, as observed in our results, makes AMSA a useful guide for DF decision.

Different approaches to VF analysis have been tested, resulting in variable accuracy for predicting DF success *(Callaham 1993, Eftestøl 2000 and 2005, Nakagawa 2012, Neurauter 2007, Watson 2006 Weaver 1985)*. Accordingly, a VF feature with at least a 50% specificity at a 95% sensitivity has been suggested as a safe and useful predictor for DF decision *(Eftestøl 2005, Neurauter 2007)*. Unlikely, results from relatively large clinical databases have demonstrated that only few of the proposed methods achieved the above limit *(Eftestøl 2005, Nakagawa 2012, Watson 2006)*. Indeed, the majority were considered with insufficient predictive power, having a high sensitivity (> 90%), but a low specificity (< 40%). Differently, AMSA, together with other VF features, i.e. median slope, power spectrum analysis, and the wavelet transform-derived Cardioversion Outcome Predictor, achieved a sensitivity of approximately 95% with a specificity between 56-66% *(Eftestøl 2005, Nakagawa 2012, Watson 2006)*. In our studies, AMSA, as predictor of DF outcome, was confirmed to yield a specificity of more than 50% at a 95% sensitivity. Moreover, different thresholds may be selected in order to maximize the DF outcome prediction, such to achieve the best PPV and the best NPV. The present study provided evidence that AMSA represents a clinically applicable tool, easily obtained from the routinely available ECG. Accordingly, the discussion attempts to suggest a potential AMSA-based algorithm for first and subsequent DF attempts.

Upon arrival at the cardiac arrest scene, rescuers need to determine the optimal first CPR intervention, i.e. a cycle of CC or an immediate DF *(AHA guidelines 2005, Baker 2008, Iwami 2012, Link 2010, Shanmugasundaram 2012, Wik 2005)*. Sun and colleagues *(Sun 2011)* have recently optimized the timing of the first DF by applying real time AMSA analysis during CC in a rat model of VF. DF was attempted only when AMSA achieved a predefined threshold predictive of successful shock. With that approach, 70% of animals were resuscitated after the first DF, in contrast to 0% when DF was attempted following standard guidelines. AMSA-guided CPR also significantly reduced CC interruptions and ultimately improved post-resuscitation myocardial and neurologic function and survival. In our population, interrupting CC for delivery of first DF at an AMSA of 16-17 mV-Hz could have raised first DF success up to 77% compared to 27% observed with the standard approach.

AMSA also has predictive ability for subsequent DF attempts, but is dependent upon the type of VF. Subsequent DFs may be due to either VF refractory to earlier DFs or to a recurrence of VF after an initially successful DF. Similar to an earlier small retrospective study on 44 patients *(Shanmugasundaram 2012)*, our study demonstrated that AMSA was accurate for DF prediction only in the instance of refractory VF. The AMSA value to be considered as a threshold for delivery of a DF with the highest likelihood of success was again 17 mV-Hz. AMSA therefore might be an useful decision tool for the treatment of refractory VF. Recurrent VF, on the other hand, presented an overall high AMSA, with no differences between successful and unsuccessful DFs, and an elevated DF success rate of almost 80%. Recurrent VF will probably not benefit from an initial interval of CC, but rather should receive DF immediately, similar to a witnessed arrest.

The total electrical energy delivered with repetitive DFs might be an important determinant for the severity of post resuscitation myocardial dysfunction and survival *(Tang 1999 and 2004 and 2006, Tsai 2011, Xie 1997)*. Using AMSA lower than 7 mV-Hz as guidance, more than 45% of unsuccessful and potentially detrimental DFs might have been avoided with a NPV > 97%. This approach could also reduce unnecessary CC interruptions for the delivery of futile DFs. Even minimal interruptions of less than 4 sec can generate declines in myocardial perfusion to near 40% *(Berg 2001)*. In our population, pre-DF pauses caused significant decreases in AMSA, likely related to the reduced myocardial perfusion and ultimately anticipating decreases in DF success *(Eftestøl T 2004, Li 2008b)*. Limiting the frequency and duration of such CC interruptions may improve clinically relevant outcomes in cardiac arrest *(Cheskes 2011, Link 2010, Steen 2003, Yu 2002)*.

Compared with other ECG-derived parameters, i.e. VF amplitude (RMS), and frequencies (PF, MDF, and MNF), whose characteristics have been described in depth in the introduction, AMSA was a better predictor of DF outcome, with an AUC that resulted equivalent in both the US and the Italian database, i.e. 0.86-0.87. Interestingly, although AMSA, au pair of other VF features, has been ideated and recognized primarily as a predictor of DF success, our results showed that it was also an independent predictor of ROSC and in patients who were successfully resuscitated it was also associated with survival till hospital discharge, and 6 months and 1 year later. Based on these results, it can be extrapolated that, patients who had higher AMSA during CPR, had probably a better myocardial perfusion and systemic hemodynamic support, due to several factors, such as duration of untreated cardiac arrest, quality of CPR, drugs, and this ultimately accounted for a better post-resuscitation survival.

Little is known about effects of various drug treatments on ECG-derived parameters. Animal models have shown that several cardiovascular drugs, i.e. beta-blockers and angiotensin converting enzyme inhibitors may alter VF features, shortening its duration and favouring the evolution towards asystole *(Wang 2009)*. More recently, the effects of pretreatment with beta-blockers on quantitative VF waveform measures have been investigated in a swine model of cardiac arrest and CPR. Indeed, metoprolol and labetalol, administered prior to VF onset, caused reduction in frequencies in the Fourier spectrum of VF and in AMSA, limiting their ability to predict downtime or DF outcome *(Sherman 2012)*. Our studies confirmed that several treatments, including anti-hypertensive, angiotensin converting enzyme inhibitors, anti-thrombotic drugs, cardiac drugs (glycosides, anti-arrhythmic drugs, inotropes), had different effects among the various parameters. More specifically, anti-hypertensive drug prescription was associated with higher AMSA values. As expected, beta-blocker treatment affected MDF and MNF, but not AMSA.

Similarly, there are very limited investigations on the relationships between VF features and co-morbidities, including and not only cardiac diseases. Swine models of cardiac arrest with concurrent AMI revealed that VF spectral features, such as median, mean, or dominant frequency and bandwidth were significantly reduced *(Indik 2007)*. However, ECG predictors, i.e. AMSA and its slope, maintained their capability to predict ROSC *(Indik 2009)*. Nevertheless, during CC, the VF waveform evolved differently, offering a means to distinguish an AMI. Indeed, AMSA and slope were significantly lower for AMI swines compared with controls, whereas in post AMI swines the waveform characteristics were similar to controls *(Indik 2011)*. Our clinical studies confirmed that cardiac disease, i.e. myocardial infarction and congestive heart failure, had impact on AMSA values. Moreover, AMSA was affected also by age, presence of diabetes, and EMS arrival time. These factors

affected in different way also the other ECG-derived parameters. Therefore, drug treatments and co-morbidities need to be taken into account when the ECG predictors are used and further studies are needed to better understand changes in AMSA thresholds in relation to these factors.

AMSA appears to carry the additional promise of being able to monitor the effectiveness of CC. The present analyses have demonstrated that AMSA decreased between consecutive shocks during shallow CC, while it increased when CC was of greater depth. We have previously reported the possibility of assessing CC depth utilizing AMSA in a porcine model, in which animals were randomized to optimal or suboptimal depth of CC *(Li 2008b)*. Similarly to CPP, AMSA thresholds achieved were contingent on the depth of compressions such that AMSA increased progressively during CC, and predicted the likelihood of successful DF. Therefore, when future technology allows for real time AMSA analyses during CC, a rescuer may be prompted to push harder if the AMSA value is too low *(Aramendi 2012, Ruiz 2013)*.

Based on our results the following AMSA algorithm to guide CPR could be beneficial. In the presence of a very low AMSA, i.e. below 7 mV-Hz, the likelihood to deliver a successful DF is low. CC therefore should be performed until AMSA is deemed to be favourable for a DF, minimizing interruptions in CC and delivery of unnecessary shocks. The decision of interrupting CC to attempt a DF should be limited to when there is the highest probability of success. DF may be delivered as initial intervention or during CC even before completion of a regular 2 min cycle when AMSA reaches the threshold of 17 mV-Hz. If AMSA values fall in the range between 7 and 17 mV-Hz, even though specificity and accuracy of shock success prediction are high, CPR should be performed accordingly to guidelines. Finally, recurrent VF should always receive an immediate DF.

We recognize important limitations. First, these were retrospective analyses. Second, AMSA was calculated only during the pre-DF hands off time and not in real time during CC. Nevertheless, for the first time effects of different factors, i.e. EMS arrival time, drugs, co-morbidities, etc. have been investigated. Moreover, we could use two large databases, that allowed for estimation of robust thresholds to be tested in future prospective evaluations.

CONCLUSIONS

Our experimental studies demonstrated that KP is activated early in the post-resuscitation and persists during the initial 3 to 5 days post-cardiac arrest, in rats, pigs, and humans. KP activation is significantly related to the severity of post-resuscitation myocardial dysfunction, cerebral injury, functional outcome and survival. The subsequent large clinical study showed that the admission levels of KP metabolites are associated with the duration of ischemia and the severity of shock during the first 24 hours in critically ill out-of-hospital cardiac arrest patients. In addition, KYNA and 3-HAA, independently predict dismal neurological outcome. Therefore, this pathway may be an important mechanism contributing to the severity of post-cardiac arrest syndrome.

The two retrospective studies on AMSA as predictor of successful DF, the largest to now, confirmed that AMSA is capable to predict DF outcome with high accuracy, for both initial and subsequent attempts. Moreover, AMSA appears as a better predictor of DF outcome compared to other ECG-derived parameters. Indeed, specific AMSA thresholds in order to predict different DF outcomes, i.e. success or failure, were identified during CPR. AMSA shows also additional promise for monitoring the long-term (up to 12 months) effectiveness of CC. An AMSA-based DF decision therefore would be an useful approach to guide the best CPR intervention. Our results provide solid data for a future prospective study, adopting a real time AMSA analysis during CPR.

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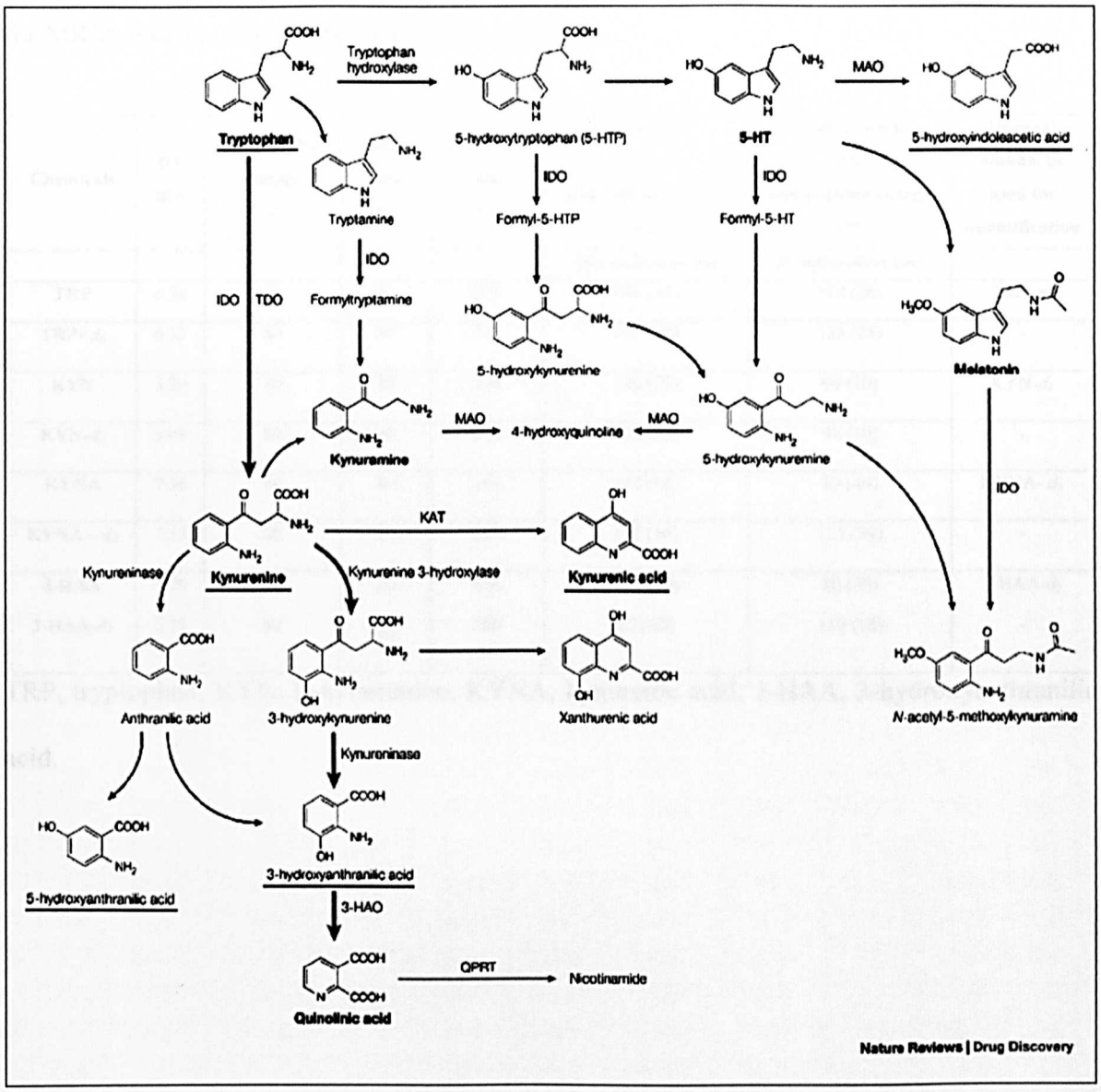
APPENDIX

Supplemental Figure 1. Key elements of the tryptophan breakdown metabolic pathway



Conversion of tryptophan to 5-hydroxytryptamine (5-HT) and to 5-hydroxyindolacetic acid and tryptophan oxidation by the kynurenine pathway. IDO indoleamine 2,3-oxidase, TDO tryptophan 2,3-dioxygenase, MAO monoamino-oxidase, KAT kynurenine aminotransferase, 3-HAO 3-hydroxyanthranilic acid oxidase, QPRT quinolinic-acid phosphoribosyl transferase. From Stone and Darlington, Nature Reviews, 2002. The underlined metabolites are those quantified by LC–MRM–MS in this study.

Supplemental Table 1. Chromatographic retention times, MS source and analyzer conditions for MRM analysis of tryptophan and its metabolites.

Chemicals	**RT** min	**Fragmentor voltage** V	**Dwell time** ms	**Precursor ion** *m/z*	**Product ion I** *m/z* **and collision energy** eV	**Product ion II** *m/z* **and collision energy** eV	**Internal standards used for quantification**
					(Quantification ion)	*(Confirmation ion)*	
TRP	6.38	80	80	205	146 (14)	118 (26)	TRP- d_8
TRP- d_8	6.32	80	80	213	151 (16)	123 (28)	-
KYN	3.20	80	80	208	146 (20)	94 (10)	KYN-d_4
KYN-d_4	3.08	80	80	213	150 (20)	98 (10)	-
KYNA	7.56	80	80	190	144 (16)	89 (46)	KYNA- d_5
KYNA – d_5	7.53	80	80	195	149 (16)	121 (34)	-
3-HAA	5.76	80	80	154	108 (18)	80 (30)	3-HAA-d_2
3-HAA-d_2	5.75	80	80	156	82 (30)	110 (18)	-

TRP, tryptophan; KYN, L-kynurenine; KYNA, kynurenic acid; 3-HAA, 3-hydroxyanthranilic acid.

Supplemental Table 2. Features detected in positive and negative ion mode showing significant changes (two-fold increase or decrease in ion intensity, coefficient of variation (CV) <10%, Welch's test p<0.01) after cardiac arrest and cardiopulmonary resuscitation.

A: POSITIVE ION												
		pool rat plasma control (CTR)			pool rat plasma 2h postresuscitation after cardiac arrest (CPR2)			Fold change			CV (%)	CV (%)
MZ	RT	Replicate 1	Replicate 2	Replicate 3	Replicate 1	Replicate 2	Replicate 3	CPR2 vs CTR	SD	p Value	pool CTR	pool CPR2
368,12723	12,92	1142	956	1140	84505923	69679236	82218251	73009	14615,345	3,40E-03	0,099	10,1
520,34033	35,05	89304918	84028770	86886578	32474211	34537438	28906686	-2,71	0,044	1,80E-05	0,03	8,9
520,34045	35,24	21831105	23001899	22305057	71068146	68863809	69736478	3,12	0,132	6,80E-06	0,026	1,6
544,34009	35,09	66107602	60758979	66496529	11375210	11806095	13623338	-5,25	0,028	3,60E-04	0,05	9,7
544,33966	35,38	8252245	8598081	7616086	63751371	60850271	65387967	7,77	0,756	3,40E-04	0,061	3,6
1039,6731	35,02	21509691	20371245	20883174	2835552	3329329	3846467	-6,27	0,029	2,70E-06	0,027	15,1
114,066	2,84	4519863	3518009	1775512	12476557	11907185	8542713	3,36	2,074	9,40E-03	0,425	19,4
521,34332	35,05	25962596	24441333	25116967	9402575	10124311	8407509	-2,70	0,045	2,10E-05	0,03	9,3
1063,67346	35,08	22041141	21707163	21391670	2686945	3053038	2105064	-8,30	0,024	2,30E-06	0,015	18,3
545,3432	35,09	20330294	18872909	20570530	3529864	3627559	4162940	-5,28	0,026	3,00E-04	0,046	9
521,34344	35,24	6268270	6588812	6370807	20588468	19990562	20262835	3,16	0,128	4,60E-06	0,026	1,5
1039,67407	35,21	3154299	3986165	2515385	16061656	15471835	15704830	4,89	1,213	2,60E-04	0,229	1,9
545,34344	35,39	2636974	2725001	2427708	20747548	19718512	19858643	7,74	0,67	1,40E-04	0,059	2,8
520,3396	35,43	8577324	8386890	7524490	38679179	32920025	40332608	4,57	0,791	5,10E-03	0,069	10,4
544,33954	35,56	3845694	3718334	3308814	38174157	34553393	39063927	10,28	1,455	1,50E-03	0,077	6,4
1063,67151	35,3	2137205	2403945	2029115	16409544	14999500	16305388	7,26	0,999	6,10E-04	0,088	4,9
1087,67188	35,41	185127	215678	156097	15434344	13934613	15087965	79,83	17,042	9,40E-04	0,16	5,3
1087,67224	35,13	16487338	17022405	16502149	2169098	2316508	1681454	-8,11	0,022	6,60E-07	0,018	16,2
369,13	12,92	5515	6705	7538	15825955	13133408	15250774	2237,58	560,77	3,10E-03	0,154	9,6
1040,67627	35,02	12444112	11940956	12031190	1665450	1936205	2269427	-6,20	0,028	1,90E-06	0,022	15,5
699,22675	27,79	0	601	0	13235660	16876225	16056242	76818,85	142480,47	5,10E-03	1,732	12,4
350,11719	14,9	0	0	682	35801045	32229321	37398062	154587,14	279404,24	1,90E-03	1,732	7,5
1064,67627	35,09	13291742	12608419	12792717	1521128	1738212	2067686	-7,26	0,025	3,20E-06	0,027	15,5
1040,67712	35,21	1706968	2198427	1382894	9374255	8885596	9143532	5,18	1,346	6,20E-05	0,233	2,7
1064,6748	35,3	1182838	1412625	1167388	9773250	8989500	9763081	7,58	1,189	4,00E-04	0,11	4,7
1088,67517	35,41	112455	119662	94664	9421504	8587428	9229213	83,35	13,856	7,80E-04	0,118	4,8
1088,67664	35,09	10049063	9507526	9863954	527486	560788	686731	-16,57	0,01	9,60E-05	0,028	14,2
194,08133	15,97	1866	1832	2114	8485918	7126157	7880534	4042,09	672,769	2,50E-03	0,079	8,7
180,06546	14,38	768744	811276	1136766	6539850	5841396	5929574	6,74	1,918	2,20E-04	0,222	6,2
426,32166	29,09	2876	2366	2648	8585358	8759251	8284228	3248,27	406,331	2,60E-04	0,097	2,8
243,1344	15,69	1141476	893781	104188	5291981	5418197	4998251	7,34	5,879	1,70E-03	0,76	4,1
223,09692	29,43	5365309	5927263	6991032	113592	99946	81034	-62,07	0,005	6,20E-03	0,135	16,7
436,19287	14,93	276084	278114	393342	6695643	7034296	6668755	21,53	5,22	9,10E-05	0,213	3
568,33966	35,05	7973664	7462650	7368047	1442478	1420630	1203635	-5,61	0,025	1,90E-04	0,043	9,7

494,32425	31,57	8038174	7910769	8070501	0	0	0	CTR only	0	3,70E-05	0,011	0
426,32175	25,56	6616199	5134227	5152919	0	0	1132	-14932,28	0	7,50E-03	0,151	173,2
340,10321	15,28	383432	394458	196444	3642850	3716612	4237384	11,90	5,076	1,10E-03	0,343	8,4
520,34003	35,61	4422871	4242681	3607998	11439106	10357484	10939843	2,67	0,411	9,80E-05	0,105	5
102,96992	2,184	160746	125820	71581	3921162	3758807	4209214	33,20	14,404	8,00E-04	0,376	5,8
177,05478	29,43	3694414	4205510	4829589	71236	63732	49116	-69,15	0,005	6,10E-03	0,134	18,3
1063,67285	35,48	221337	170792	155878	10838588	8808034	10584795	55,17	16,418	4,10E-03	0,188	11
521,34283	35,43	2452182	2426374	2146187	11111378	9439271	11568066	4,57	0,81	5,10E-03	0,072	10,5
105,00314	2,217	57177	36771	23159	3056788	3209822	3624529	84,46	44,572	2,60E-03	0,439	8,9
544,33997	35,75	47930538	47255157	37493238	8298301	7514252	10325688	-5,08	0,059	6,30E-03	0,132	16,7
854,35193	14,93	6909	8529	8858	3026732	3060789	2877497	368,99	59,591	3,60E-04	0,129	3,3
568,33984	35,34	858200	873119	789059	4975405	4721953	4808165	5,76	0,461	8,40E-05	0,053	2,7
1041,67932	35,02	3834531	3606349	3622483	502709	576272	674234	-6,31	0,029	1,40E-05	0,035	14,7
496,33948	35,54	4137005	3291718	4435254	122591	124787	115504	-32,69	0,006	7,90E-03	0,15	4
545,34351	35,58	1060843	1076903	930777	9703394	8745055	9931910	9,25	1,34	1,60E-03	0,078	6,7
350,11719	15,08	0	0	794	7695188	8875262	8031581	30984,93	56002,026	1,80E-03	1,732	7,4
125,98596	2,183	110720	90980	51329	3021296	2892183	2884658	34,77	13,38	3,70E-05	0,359	2,6
225,12376	15,69	524325	438691	47075	2776616	2668080	2603617	7,97	6,281	1,70E-03	0,756	3,3
1065,67908	35,09	4112757	3896340	3849053	431191	513696	605369	-7,65	0,027	1,90E-05	0,036	16,9
404,20688	28,94	3696319	3662151	3654663	0	0	0	only CTR	0	1,20E-05	0,006	0
522,34607	35,05	4084239	3950376	4015049	1555514	1665762	1386663	-2,61	0,041	1,50E-04	0,017	9,2
388,60629	14,23	0	180	0	2686340	2712114	2689541	44933,31	78035	9,00E-06	1,732	0,5
347,22183	26,72	591549	0	0	3328275	3232029	3329940	16,72	29,243	3,30E-03	1,732	1,7
110,00856	2,217	19928	13775	7486	2266392	2284166	2327429	166,99	77,951	3,40E-05	0,453	1,4
520,34039	35,79	21831105	20592598	19483800	5111785	5346344	5309598	-3,93	0,021	1,70E-03	0,057	2,4
546,34515	35,09	3423117	3060703	3439476	523652	528652	608308	-5,98	0,025	1,30E-03	0,065	8,6
432,23877	28,95	3798696	3643869	3728066	225	350	0	-19427,18	0	1,40E-04	0,021	92,5
130,96486	2,184	45211	40339	18034	2345870	2117302	2490044	67,13	33,615	2,10E-03	0,42	8,1
1089,67944	35,09	3121391	2931977	3150260	151249	189589	216321	-16,52	0,013	2,40E-04	0,039	17,6
1041,68005	35,21	492942	621273	361232	2834588	2636679	2721333	5,55	1,67	3,20E-05	0,264	3,6
494,32462	33,72	685498	688224	683490	4387576	5740267	5387868	7,54	1,049	8,10E-03	0,003	13,6
1089,6781	35,41	31304	33886	27972	2992903	2733153	2892454	92,51	13,05	7,00E-04	0,095	4,6
243,13417	14,9	628602	619306	871592	2811046	2680025	2716190	3,87	0,88	2,80E-04	0,202	2,5
1065,67761	35,3	356156	416192	336787	2989398	2672985	2920927	7,74	1,317	8,40E-04	0,112	5,8
468,30832	30,77	3203352	3010396	3096461	0	0	0	only CTR	0	3,20E-04	0,031	0
404,20724	30,97	8536	10198	9657	2950601	2997698	2939470	313,05	31,301	3,60E-05	0,09	1
436,19287	13,91	110280	103684	110988	2371007	2528131	2303996	22,17	1,886	8,30E-04	0,037	4,8
546,34619	35,39	398302	411324	357700	3438072	3368529	3360295	8,71	0,736	4,00E-07	0,072	1,3
1064,67615	35,48	118759	98867	91522	6421122	5188432	6280180	57,87	14,461	4,40E-03	0,137	11,3
522,34558	35,28	799121	777606	610546	3291690	3063047	3127321	4,34	0,775	1,30E-05	0,142	3,7
211,108	15,99	421	272	0	2055053	1997993	1848498	8515,94	8333,375	9,80E-04	0,924	5,4
214,08987	2,009	121532	655508	75062	3559183	4195277	4054177	13,86	16,913	1,70E-04	1,136	8,5
128,01924	2,217	36035	24589	14758	1709951	1755481	1795189	69,79	31,272	9,00E-05	0,424	2,4
419,16629	14,93	70324	75913	95550	2394409	2314660	2256070	28,81	5,597	2,00E-04	0,164	3

132,99818	2,217	12911	8781	4363	1680906	1541280	1846103	194,52	113,323	2,70E-03	0,492	9
370,12347	11,96	446	0	0	1968786	2013049	1999403	13410,85	23399,352	4,30E-05	1,732	1,1
373,27405	29,09	474	402	673	2299380	2051052	2253902	4263,61	1414,493	1,20E-03	0,272	6
229,15498	4,398	126694	151051	101025	2700895	2487265	2539120	20,40	4,925	4,00E-04	0,198	4,3
432,23834	31	5543	6383	7044	2281147	2233977	2282884	358,36	47,006	5,00E-05	0,119	1,2
1049,33911	25,41	0	0	424	1779847	1743227	1725654	12379,08	21664,577	8,30E-05	1,732	1,6
427,32489	29,09	0	0	330	2255067	2304722	2162010	20369,09	35949,839	3,50E-04	1,732	3,2
569,34247	35,06	2404959	2229885	2256210	392201	408943	336580	-6,06	0,023	1,70E-04	0,041	10
524,67188	25,05	626	0	0	2143131	2289644	2219464	10626,58	18758,549	3,60E-04	1,732	3,3
1111,67224	35,09	1956452	1877992	1910555	95443	104397	131127	-17,36	0,011	1,00E-05	0,021	16,8
148,97548	2,184	49333	39910	21435	1581653	1389011	1634969	41,61	19,515	2,20E-03	0,385	8,4
338,08722	14,16	274185	282383	301817	1434157	1453236	1489506	5,10	0,351	9,40E-06	0,05	1,9
387,18082	28,96	1963785	1935670	1878275	0	0	0	only CTR	0	1,70E-04	0,023	0
834,60974	25,56	1417914	1136019	1123540	0	0	0	only CTR	0	6,10E-03	0,136	0
1039,67407	35,39	286253	293017	212766	10018860	8502848	8308820	33,88	9,257	3,80E-03	0,169	10,5
420,19794	20,26	827	802	496	1702250	1641116	1682302	2365,02	658,542	1,20E-04	0,26	1,9
368,15536	15,27	4130	7899	6027	1455248	1594345	1603294	257,69	94,477	9,60E-04	0,313	5,4
401,15552	14,9	26284	28349	36343	1451512	1409000	1384673	46,66	9,291	1,40E-04	0,175	2,4
190,08635	20,47	1044516	1437437	1371570	13781	15317	15186	-87,02	0,003	9,00E-03	0,164	5,8
495,32782	33,8	519883	523192	569144	1572690	1908906	1835891	3,30	0,498	5,80E-03	0,051	10
518,32434	31,39	1914198	1881131	1797706	0	0	0	only CTR	0	3,50E-04	0,032	0
495,32745	31,57	2130141	2098017	2150851	0	0	148	-43101,41	0	5,20E-05	0,013	173,2
427,32495	25,56	1734329	1333099	1351240	0	0	0	only CTR	0	7,80E-03	0,154	0
241,11839	16,62	0	519	479	1037723	1259532	1292011	3596,46	3535,983	4,40E-03	0,868	11,6
380,25616	29,62	1230680	947176	1028342	0	0	0	only CTR	0	6,20E-03	0,137	0
268,15479	29,43	1748461	1794194	2017893	23952	22459	21051	-82,42	0,002	2,10E-03	0,078	6,5
874,28339	31,75	0	0	412	2741460	2749914	2518067	19440,39	34606,588	8,10E-04	1,732	4,9
350,11719	15,25	0	0	230	3268495	3441567	3194280	43062,36	76396,109	4,90E-04	1,732	3,8
478,29269	34,77	1712368	1646819	1593193	719316	705970	627912	-2,41	0,045	3,60E-05	0,036	7,2
521,34326	35,61	1238458	1194682	1010000	3221582	2979673	3088560	2,70	0,391	3,90E-05	0,106	3,9
373,27402	25,53	1475038	1511588	1637052	2567	340	2096	-924,18	0,001	1,00E-03	0,055	70,4
568,33936	34,86	1943079	1767430	1665672	856671	861215	904721	-2,05	0,053	6,20E-03	0,078	3
120,98051	2,184	71731	57924	36080	1119732	1127987	1218154	20,91	7,793	2,80E-04	0,325	4,7
855,35529	14,93	3161	1920	2863	1130355	1102458	1049351	413,16	116,587	4,70E-04	0,245	3,8
387,18036	30,94	41250	49851	50512	1542483	1620389	1601340	33,64	4,543	1,90E-04	0,109	2,6
588,86029	27,94	0	0	169	1938226	2100454	2096215	36301,15	64385,175	6,80E-04	1,732	4,5
232,15448	10,38	74385	497667	11644	1766245	1596237	1285914	7,96	12,072	2,90E-03	1,359	15,7
548,37061	39,33	831853	665403	415797	2079512	1877241	1912965	3,07	1,177	2,40E-03	0,328	5,5
380,25635	30,52	1570238	1659378	1607545	0	1529	1405	-1648,66	0,001	2,60E-04	0,028	86,8
260,16086	15,69	236634	172885	26307	951346	1072340	889121	6,68	5,603	6,20E-04	0,742	9,6
214,09015	2,217	50510	28370	19986	2176765	1824628	1770424	58,38	34,631	4,30E-03	0,479	11,5
1111,67175	35,35	42633	47998	35061	1302433	1242798	1303342	30,62	5,578	1,70E-04	0,155	2,7
489,31436	18,97	10818	12785	10048	904875	899843	732700	75,40	18,223	4,60E-03	0,126	11,6
316,1395	19,54	0	0	222705	926218	975265	822083	12,23	22,236	1,60E-03	1,732	8,6

261,14478	12,23	318546	310272	297339	1707053	1451624	1565187	5,10	0,591	3,20E-03	0,035	8,1
1048,33752	31,66	187	0	0	1918459	2070415	1834862	31142,97	55760,242	1,30E-03	1,732	6,2
437,19598	14,93	41629	47991	60120	1217806	1263434	1217605	24,70	5,178	3,20E-05	0,188	2,1
1112,67664	35,09	1176016	1160217	1189784	60118	63991	75668	-17,65	0,008	1,00E-06	0,013	12,2
569,34192	35,3	286001	298703	281676	1327684	1277342	1362584	4,58	0,289	3,60E-04	0,031	3,2
701,22235	14,57	0	0	268	1372512	1309451	1234527	14613,77	26109,086	9,30E-04	1,732	5,3
525,17261	25,1	275	0	0	1239286	1039897	1270238	12906,99	23711,933	3,70E-03	1,732	10,6
542,32111	34,98	1425042	1347525	1397544	544928	633986	447070	-2,56	0,078	1,30E-03	0,028	17,2
502,29309	35,02	179147	214916	222383	1182920	1143767	1090477	5,54	0,849	8,50E-05	0,112	4,1
156,01428	2,217	6345	5160	2351	841953	864747	916320	189,31	92,329	6,20E-04	0,444	4,4
216,9511	2,217	22808	18014	11379	914317	831333	912898	50,93	19,528	8,60E-04	0,33	5,4
489,31427	17,01	15988	19083	16921	0	0	0	only CTR	0	2,80E-03	0,092	0
520,33893	36,49	1884186	1763071	1546908	931461	848171	753437	-2,05	0,1	4,10E-03	0,099	10,5
545,34351	35,79	10369307	10343340	9611180	1774686	1960049	2047860	-5,24	0,022	3,30E-04	0,043	7,2
873,7818	29,02	0	209	0	1332324	1163105	1259993	17968,53	32344,243	1,50E-03	1,732	6,8
473,49094	25,72	0	0	223	901620	944941	961808	12593,58	22213,373	3,70E-04	1,732	3,3
542,32416	31,3	1173127	1167305	1128671	0	0	0	only CTR	0	1,50E-04	0,021	0
322,13214	19,61	0	0	13015	846042	788829	893404	194,26	348,532	1,10E-03	1,732	6,2
874,28253	32,34	221	0	0	1682188	1751017	1903185	24146,56	43323,473	1,30E-03	1,732	6,4
350,11734	15,47	322	0	0	1515943	1442203	1792643	14754,00	27285,121	4,50E-03	1,732	11,7
510,35532	39,36	783820	638582	335170	2221304	1659224	1686922	3,17	1,779	6,50E-03	0,391	17,1
171,99159	2,183	28164	23981	12651	790139	761269	802085	36,32	14,469	4,30E-05	0,372	2,7
542,32178	35,21	605531	610118	571601	1307507	1281020	1231478	2,14	0,14	9,30E-05	0,035	3
467,30133	16,96	11675	15209	13207	0	0	0	only CTR	0	5,80E-03	0,133	0
341,10648	15,28	60375	59238	31611	605033	625433	712278	12,85	5,281	1,60E-03	0,323	8,8
874,28363	30,7	207	0	0	2258248	2672126	2968454	38158,59	71199,325	6,10E-03	1,732	13,5
497,34274	35,54	1092475	861957	1172523	25479	23697	24992	-42,16	0,005	8,30E-03	0,155	3,7
1040,67529	35,41	129543	133265	96995	4457555	3513584	4704732	35,23	11,103	7,70E-03	0,166	14,9
835,61383	25,52	655355	719643	723484	0	0	0	only CTR	0	1,00E-03	0,055	0
327,00864	30,76	1477170	1418180	1501942	336901	277719	316125	-4,72	0,027	8,50E-06	0,029	9,7
511,32687	19,15	7905	7442	5280	588297	680007	582735	89,74	26,236	2,60E-03	0,204	8,9
313,17584	3,737	189750	283596	220085	507574	506658	510445	2,20	0,464	9,70E-03	0,207	0,4
368,15512	15,5	7929	9979	7464	1087976	1023891	909885	119,10	29,508	2,70E-03	0,158	9
1112,67566	35,35	26068	33160	24093	840091	752640	797477	28,69	6,5	9,60E-04	0,172	5,5
395,1748	14,7	743	510	0	1383206	1136510	1075438	2869,24	3001,458	6,10E-03	0,91	13,6
190,04996	13,17	3401	1443	2287	1017756	963811	959662	412,46	184,08	3,60E-04	0,413	3,3
582,85693	31,22	155	0	0	1641859	1523577	1797105	32016,39	57963,998	2,30E-03	1,732	8,3
329,00543	30,74	1363347	1278694	1388895	290144	286944	271217	-4,75	0,017	7,20E-04	0,043	3,6
1063,67261	34,83	1018875	886709	793100	396768	346806	312551	-2,56	0,096	7,60E-03	0,126	12
239,13925	15,97	325	0	524	979386	801282	887886	3143,17	3253,066	3,30E-03	0,935	10
195,08461	15,99	407	0	0	917823	908799	853789	6585,78	11669,012	5,00E-04	1,732	3,9
391,28482	29,07	363389	394583	405818	959096	886101	938892	2,39	0,233	1,40E-04	0,057	4,1
224,10011	29,43	703365	773325	908169	9227	8687	7536	-93,71	0,002	5,80E-03	0,131	10,2
115,06929	2,84	145808	98729	56992	437347	435669	322812	3,97	2,406	4,40E-03	0,442	16,5

464,30118	23,87	0	0	211	645895	710894	660766	9561,87	17050,065	8,50E-04	1,732	5,1
874,69891	24,84	389954	23264	430742	1222636	1390016	1172990	4,49	3,982	6,90E-03	0,798	9
244,13762	15,69	132454	99776	7094	616473	637903	607603	7,78	6,537	3,20E-03	0,815	2,5
521,34369	35,79	6268270	5886418	5576289	1447309	1527101	1504726	-3,96	0,022	1,80E-03	0,059	2,8
566,32104	35,45	321282	331647	282287	1083389	1139497	1066440	3,52	0,416	2,40E-05	0,083	3,5
874,6991	22,71	1227392	1064799	903972	0	0	0	only CTR	0	7,60E-03	0,152	0
405,21085	28,95	889728	855848	884671	0	0	0	only CTR	0	1,40E-04	0,021	0
214,91762	2,184	37403	35573	20501	562270	518326	543932	17,38	5,882	1,00E-04	0,298	4,1
874,44824	24,84	358302	25243	433736	1152245	1327690	1137449	4,43	3,92	7,60E-03	0,798	8,8
1104,42688	28,79	105778	106413	112829	832593	1051773	1051586	9,03	1,493	6,90E-03	0,036	12,9
288,10809	13,44	10775	8383	5481	843465	936400	883268	108,09	40,571	9,00E-04	0,323	5,3
410,32657	34,46	938938	833687	827023	108424	91345	84404	-9,15	0,022	1,60E-03	0,072	13,1
114,98752	2,217	1901	1394	617	562718	530055	617294	437,13	250,588	2,00E-03	0,496	7,7
181,06865	14,38	74083	79404	106026	610908	542577	541493	6,53	1,752	5,60E-04	0,198	7
433,242	28,95	998471	966289	972391	0	0	0	only CTR	0	1,00E-04	0,017	0
566,3208	35,12	1084213	968287	1055056	277121	275663	305573	-3,62	0,032	1,10E-03	0,058	5,9
149,02325	29,43	645564	862628	860449	86187	78825	71486	-10,02	0,025	9,90E-03	0,158	9,3
1066,68274	35,09	866665	837518	825545	97949	118922	139879	-7,09	0,028	1,90E-06	0,025	17,6
1065,67908	35,48	37292	31103	26671	1965381	1511170	1887967	56,43	17,168	6,30E-03	0,168	13,6
874,44812	22,72	1245543	1032398	942961	0	382	0	-8431,68	0	6,90E-03	0,145	173,2
348,22507	26,72	126515	0	0	746908	729300	753113	17,62	30,813	2,90E-03	1,732	1,7
874,28357	32,56	207	0	0	1158707	1351189	1320511	18504,38	33519,599	2,20E-03	1,732	8,1
468,30856	32,48	107660	91282	176339	2315747	2417467	2351057	18,88	7,223	7,30E-07	0,361	2,2
229,15486	3,613	78931	96384	103499	1388394	1242360	999604	13,02	3,884	9,90E-03	0,136	16,2
260,16083	14,97	465560	422829	505342	72507	61095	54126	-7,42	0,032	2,40E-03	0,089	14,8
196,06053	10,53	0	3005	0	789807	686350	608338	693,68	1292,252	5,70E-03	1,732	13,1
246,1701	14,07	127470	144415	194614	707254	712924	649607	4,44	1,222	4,80E-05	0,225	5,1
1042,68237	35,05	760367	771784	752229	263530	280977	217316	-3,00	0,048	6,20E-04	0,013	13
206,08124	18,81	120651	0	0	555014	582585	492876	13,51	24,549	9,70E-04	1,732	8,5
502,28729	15,69	47322	27999	388	436274	483362	389389	17,29	18,023	9,50E-04	0,935	10,8
426,32138	28,91	3200	1647	2406	3584133	3970545	4757159	1697,48	792,595	7,00E-03	0,321	14,6
157,06073	15,69	97470	84267	5368	539185	538804	517696	8,53	7,007	2,40E-03	0,799	2,3
485,19897	3,396	149576	154907	136149	56450	46434	9327	-3,93	0,186	9,10E-03	0,066	66,4
469,31158	30,77	773915	721052	735844	0	0	0	only CTR	0	4,50E-04	0,037	0
372,06668	30,77	1103021	1020242	1069678	72453	55479	66265	-16,44	0,01	3,60E-04	0,039	13,3
531,24939	16,01	0	0	182	456875	430251	428652	7229,55	12818,27	4,40E-04	1,732	3,6
568,33972	35,52	306226	316216	269721	2451065	2180304	2019311	7,45	1,347	3,90E-03	0,082	9,8
405,21042	31	696	1632	1426	672694	663095	681903	537,48	218,704	6,30E-05	0,393	1,4
1090,68237	35,09	699615	647680	695779	37333	44990	50192	-15,42	0,012	4,00E-04	0,042	14,6
357,27887	34,5	735162	748734	687805	165554	108302	137097	-5,28	0,048	2,10E-05	0,044	20,9
384,11508	10,46	0	9935	0	768407	878641	803547	246,66	444,252	1,40E-03	1,732	6,9
426,32132	26,36	7201	4283	4980	538704	512380	552541	97,40	30,769	4,60E-04	0,278	3,8
322,09238	16,08	0	0	250	423341	457529	488542	5477,65	9880,994	1,70E-03	1,732	7,1
943,97382	25,77	0	0	80	548508	490997	544473	19799,73	35674,945	1,20E-03	1,732	6,1

174,11238	15,66	11021	17267	11507	520370	580731	475168	39,61	14,368	3,40E-03	0,262	10,1
391,28445	25,53	756069	765970	805400	270600	253913	282815	-2,88	0,03	6,30E-05	0,034	5,4
699,56085	31,01	253	0	0	701352	725070	709366	8441,85	14758,325	9,60E-05	1,732	1,7
374,06339	30,76	1006126	913644	1059225	71430	54838	65589	-15,53	0,013	1,90E-03	0,074	13,2
295,12906	13,57	140435	136735	120555	630695	607207	576020	4,56	0,571	2,90E-04	0,08	4,5
485,2608	15,69	61121	34343	455	388909	387547	368962	11,94	11,704	8,90E-04	0,951	2,9
143,99655	2,183	32830	28063	16775	468405	496735	407464	17,67	7,39	2,90E-03	0,318	10
874,28369	30,52	428	0	0	1152056	1186216	1218691	8310,66	14615,73	2,60E-04	1,732	2,8
327,00824	30,93	1083958	1151671	1234896	310964	275755	280031	-4,00	0,033	1,50E-03	0,065	6,6
107,00066	2,217	7060	4178	2463	474020	455329	514166	105,36	60,175	1,20E-03	0,509	6,2
1090,68103	35,41	5193	8509	6280	638936	576473	623602	92,03	28,249	9,30E-04	0,254	5,3
174,11247	16,25	2838	374	2017	456967	506326	551486	289,69	235,688	2,90E-03	0,72	9,4
274,13995	15,77	429335	360476	325538	130381	162980	198343	-2,27	0,154	7,30E-03	0,142	20,7
245,07883	29,43	557707	654260	698561	2220	2277	621	-373,30	0,002	4,30E-03	0,113	55,1
218,98413	2,217	2488	1223	1012	416249	366354	407942	252,07	144,869	1,50E-03	0,507	6,7
546,34619	35,58	134652	137669	119869	1664551	1500745	1711388	12,43	1,752	1,70E-03	0,073	6,8
329,00549	30,93	1150935	1132832	1105218	282176	282355	290884	-3,96	0,01	1,40E-04	0,02	1,7
874,94946	24,84	268824	13268	304965	881359	971297	829234	4,57	4,079	7,80E-03	0,813	8
340,10312	15,47	656720	326943	557488	2249822	1777077	2570736	4,28	2,187	9,30E-03	0,329	18,2
1066,68164	35,35	31703	36869	29044	693418	605349	651602	19,98	3,797	1,60E-03	0,122	6,8
489,81589	18,97	4964	3879	4781	443436	407573	334575	87,02	23,344	6,70E-03	0,128	14
518,32416	31,16	750778	716900	738905	0	0	0	only CTR	0	1,80E-04	0,023	0
1087,67322	35,59	29292	28641	21697	5450533	4425041	5688370	195,45	56,291	5,60E-03	0,159	12,9
997,62531	34,77	513620	492134	408480	129844	109294	98783	-4,19	0,062	5,00E-03	0,118	14
331,05408	4,095	241810	377164	168205	990242	800779	884096	3,40	1,735	1,60E-03	0,404	10,6
1104,30017	27,92	7525	12613	14227	839904	924169	751280	73,20	29,897	3,60E-03	0,305	10,3
372,23837	13,34	13614	13314	8785	402685	383361	388481	32,89	8,316	9,10E-05	0,227	2,6
1104,42615	28,12	35533	92992	72862	996648	910987	725589	13,08	7,743	7,50E-03	0,434	15,8
200,9735	2,217	1740	1352	923	401351	365069	404389	291,61	105,354	1,00E-03	0,305	5,6
1042,68201	35,25	57497	56206	36455	615809	554801	583494	11,68	3,362	2,70E-04	0,236	5,2
1104,55078	27,82	9362	13970	15238	672046	897716	809909	61,70	23,689	7,00E-03	0,241	14,3
274,16537	21,97	1627	485	855	433254	421807	389902	419,60	269,846	9,70E-04	0,589	5,4
571,35889	37,32	654497	636336	498824	129315	153045	112471	-4,53	0,066	8,10E-03	0,143	15,5
445,28796	16,95	4801	5706	4647	0	0	0	only CTR	0	4,20E-03	0,113	0
874,94952	22,71	863295	753760	668797	0	0	0	only CTR	0	5,40E-03	0,128	0
106,06187	14,55	2781	2697	3173	1276371	1301124	1004263	414,03	93,616	6,30E-03	0,088	13,8
514,28284	20,65	0	0	183	305754	345104	375867	5610,52	10315,217	3,50E-03	1,732	10,3
105,03313	14,38	61155	61453	88592	435614	332277	378732	5,43	1,95	5,50E-03	0,224	13,5
106,00391	2,217	5588	3811	2465	415647	407327	439488	106,41	46,367	4,80E-04	0,396	4
511,35858	39,25	404277	330982	223925	918388	761562	748201	2,53	1,014	3,00E-03	0,284	11,7
374,27747	29,09	0	84	0	572901	490258	560878	19333,77	35263,746	2,30E-03	1,732	8,2
533,32532	20,49	881	0	345	429663	385498	359319	957,98	1127,863	2,70E-03	1,087	9,1
1104,55139	28,88	91244	98573	89624	696232	816991	796712	8,27	1,117	2,90E-03	0,051	8,4
524,67181	25,23	311	0	0	4631552	3536837	3531889	37621,47	71332,17	8,70E-03	1,732	16,2

1104,55127	28,13	35402	87710	194839	863244	790432	671755	7,31	6,521	9,00E-04	0,767	12,5
874,69934	22,98	723849	796529	746171	0	0	0	only CTR	0	8,10E-04	0,049	0
1104,30188	28,21	90925	196898	168669	837697	905972	728586	5,42	2,541	9,80E-04	0,361	10,9
503,32971	18,97	1667	1897	2303	340783	358092	312897	172,45	40,042	1,50E-03	0,165	6,8
1104,67651	28,76	74160	88410	82369	731497	668632	694818	8,55	1,136	5,20E-04	0,088	4,5
480,80099	18,97	3504	4115	5415	349608	355578	310828	77,95	23,105	1,70E-03	0,225	7,2
489,81573	17,01	7081	8394	6673	0	0	0	only CTR	0	4,90E-03	0,122	0
433,24191	30,97	1451	1025	1663	559697	563731	561005	406,97	97,449	2,70E-06	0,236	0,4
1104,17554	27,97	9176	11753	26764	693210	732137	553470	41,49	30,699	6,60E-03	0,597	14,2
243,13419	22,42	408662	443333	468086	0	808	1589	-550,72	0,002	1,50E-03	0,068	99,5
370,13379	11,96	327	273	0	475997	430143	449702	2259,74	2099,495	8,60E-04	0,877	5,1
137,07057	2,942	20131	17080	15038	474911	413904	338761	23,49	7,374	9,90E-03	0,147	16,7
437,19608	13,91	14985	12869	12990	423490	448552	407200	31,32	4,264	8,20E-04	0,087	4,9
134,05984	13,52	524527	520516	559323	34678	961	0	only CTR	0,038	6,70E-06	0,04	166,2
511,82855	19,15	3843	3543	2479	282715	328911	286122	91,00	27,657	2,50E-03	0,218	8,6
503,32962	17	5313	6953	5980	0	0	0	only CTR	0	6,10E-03	0,136	0
224,09525	13,82	19466	15301	20471	490828	556205	447597	27,06	6,998	4,20E-03	0,149	11
208,06059	13,9	7028	8371	5833	453662	326972	399265	55,57	18,95	8,90E-03	0,179	16,2
854,35187	13,91	0	945	0	342522	334241	318449	1053,13	1863,735	4,40E-04	1,732	3,7
387,18039	31,13	11174	11866	10701	1243814	978292	1366793	106,37	23,193	9,20E-03	0,052	16,6
151,03529	2,217	4216	3115	1663	345729	315827	320019	109,14	52,01	7,80E-04	0,427	4,9
246,16989	14,46	57719	55486	67335	337035	344453	353073	5,73	0,733	2,00E-06	0,105	2,3
370,1235	13,1	889	330	0	1648882	1344128	1516715	3699,53	4464,647	3,40E-03	1,105	10,2
569,34277	34,86	571541	533361	492074	242095	241968	253884	-2,16	0,047	5,30E-03	0,075	2,8
178,05803	29,43	382335	437717	509473	2686	3247	1435	-180,45	0,003	6,90E-03	0,144	37,8
250,08018	3,401	150063	153039	141545	27660	28722	5842	-7,15	0,093	8,20E-04	0,04	62,3
519,32764	31,39	532845	519364	506554	0	0	0	only CTR	0	2,10E-04	0,025	0
679,19824	15,32	3383	3536	4090	234578	207598	225003	60,60	9,869	1,30E-03	0,101	6,2
817,58258	25,53	332114	333312	347656	0	0	314	-3226,38	0,001	2,20E-04	0,026	173,2
225,12337	14,38	50847	50075	83203	306285	238188	241237	4,27	1,941	4,40E-03	0,308	14,7
494,32364	31,21	562864	525889	559231	0	0	0	only CTR	0	4,60E-04	0,037	0
874,28345	29,64	221	0	0	1059135	1081708	1009710	14255,90	25171,64	4,10E-04	1,732	3,5
232,92825	2,184	26629	22009	14789	296985	247553	309153	13,46	5,342	4,00E-03	0,282	11,5
331,05405	4,272	237047	264527	191564	834137	666021	765391	3,27	0,887	3,30E-03	0,159	11,2
261,14478	12,04	50574	56441	186714	984163	852445	994413	9,64	8,385	1,90E-04	0,786	8,4
478,29297	34,95	115499	205932	150120	760952	717065	741786	4,71	1,506	3,50E-04	0,29	3
388,18423	28,96	460907	442856	439428	0	0	0	only CTR	0	2,20E-04	0,026	0
263,19696	3,747	87739	98863	85832	297872	246704	286539	3,05	0,532	4,50E-03	0,077	9,7
701,22217	14,76	0	0	529	795171	711876	770798	4305,95	7694,854	1,10E-03	1,732	5,6
355,26349	25,53	390346	399050	445552	1549	0	600	-574,66	0,002	1,70E-03	0,072	109
502,29282	34,83	913253	760999	703620	364318	403060	233539	-2,38	0,17	5,30E-03	0,137	26,6
327,00894	31,14	863455	864766	818614	300421	290117	323307	-2,79	0,031	2,80E-05	0,031	5,6
652,41132	17,24	850	1141	0	277755	268536	236598	393,21	383,701	2,30E-03	0,893	8,3
574,19696	28,44	0	0	254	587592	513201	579150	6613,95	11943,772	1,80E-03	1,732	7,3

382,27176	33,09	175650	147757	165197	371029	451570	443490	2,59	0,497	5,60E-03	0,087	10,5
549,3739	39,33	238257	183795	106133	629959	564338	573323	3,35	1,464	2,30E-03	0,377	6
533,32574	20,28	1454	0	1129	325122	324313	343116	384,26	352,83	3,20E-04	0,886	3,2
1189,15686	25,26	736701	642780	646339	0	596	2394	-677,53	0,002	2,10E-03	0,079	125
1189,01392	25,36	729184	529930	650637	467	2513	2704	-335,99	0,002	8,20E-03	0,158	65,5
226,12708	15,69	56990	46949	3168	308843	319294	309203	8,75	7,181	2,60E-03	0,802	1,9
374,06329	30,95	768691	756388	753824	47860	46209	42387	-16,70	0,004	5,70E-06	0,01	6,2
387,18088	29,35	349059	375324	409140	137966	167902	175618	-2,35	0,086	9,70E-04	0,08	12,4
102,03347	2,183	31344	25132	13920	307390	345726	312913	13,72	6,048	3,50E-04	0,376	6,4
329,00534	31,11	759295	786626	806046	285511	292021	297961	-2,69	0,019	3,60E-04	0,03	2,1
392,14883	11,1	0	6638	363	398680	308027	306839	144,77	254,064	7,90E-03	1,599	15,6
372,06641	30,96	779988	744371	745632	58496	45256	54162	-14,37	0,011	7,00E-05	0,027	12,8
1104,67603	28,13	21225	70064	129487	688542	620154	496679	8,18	7,346	3,20E-03	0,737	16,2
1189,01379	25,17	777317	609075	731891	621	896	1399	-726,43	0,001	5,00E-03	0,123	40,6
130,15878	13,3	164451	138113	179746	349181	357010	318614	2,12	0,405	4,30E-04	0,131	5,9
376,12393	10,38	1922	20666	308	485557	494994	377982	59,33	96,499	5,80E-03	1,483	14,4
874,19806	22,71	606273	535922	447966	0	0	0	only CTR	0	7,40E-03	0,15	0
236,99837	3,023	269076	225419	204994	91351	97191	64657	-2,76	0,125	5,80E-03	0,14	20,6
374,27722	25,53	359573	373854	402064	0	0	0	only CTR	0	1,10E-03	0,057	0
874,19781	24,84	159529	12290	196221	576804	671085	573729	4,95	4,378	4,10E-03	0,793	9,1
516,30591	31,59	515985	501734	473715	210	355	0	-2639,71	0	6,20E-04	0,043	94,8
570,35492	35,47	469496	432402	404709	0	0	0	only CTR	0	1,90E-03	0,075	0
112,00609	2,217	1646	1045	683	279919	230007	277962	233,52	126,203	3,90E-03	0,433	10,8
157,08588	17,47	35870	9228	9007	506713	424395	477874	26,04	24,619	1,00E-03	0,856	8,9
175,09656	17,47	42347	8803	8715	511518	448959	489864	24,23	25,135	1,10E-04	0,972	6,6
1104,17688	28,21	61186	156314	123561	652790	668152	547982	5,48	2,905	6,30E-04	0,425	10,5
1119,40247	20,18	0	452	0	249516	263339	256075	1701,17	2990,098	2,40E-04	1,732	2,7
479,29602	34,77	395618	375629	351444	147264	144070	129889	-2,67	0,047	8,50E-04	0,059	6,6
1104,17627	28,92	59474	69090	65166	581879	470103	633767	8,70	1,947	9,10E-03	0,075	14,9
245,16106	3,808	9265	8144	2021	308838	322247	290173	47,41	31,029	5,30E-04	0,602	5,2
1113,67944	35,08	380755	359917	388927	21205	20472	15027	-19,92	0,011	3,30E-04	0,04	17,9
1189,30017	25,31	650541	487879	515137	240	223	567	-1605,40	0	8,20E-03	0,158	56,4
1048,33691	25,05	169	0	0	383260	342656	351894	6377,57	11443,18	1,20E-03	1,732	5,9
748,4115	22,79	404	0	0	272457	267507	248612	1951,92	3471,6	7,60E-04	1,732	4,8
106,06184	14,72	3218	2948	1831	984811	885470	963203	354,32	117,297	1,00E-03	0,276	5,5
522,34644	35,48	272605	246552	242428	1007605	857737	1027834	3,80	0,611	4,60E-03	0,064	9,6
388,22342	31,42	409960	416697	435734	0	0	0	only CTR	0	3,40E-04	0,032	0
874,94989	22,98	500732	589925	529750	0	326	0	-4970,57	0	2,40E-03	0,084	173,2
378,2402	29,96	189780	154219	134956	330478	373483	368672	2,24	0,538	8,10E-04	0,174	6,6
241,11855	15,97	288084	283853	299483	0	573	0	-1520,80	0,001	2,50E-04	0,028	173,2
1189,15674	25,44	616838	477832	508161	1086	1587	2742	-296,00	0,002	6,20E-03	0,137	47
520,33881	36,66	1018401	875796	802365	433661	391869	321912	-2,35	0,115	5,50E-03	0,122	14,8
526,17102	32,92	167	0	0	565578	515084	540802	9709,37	17260,243	7,30E-04	1,732	4,7
482,36105	38,11	108335	171314	189893	559249	734404	633565	4,10	1,683	3,80E-03	0,273	13,7

375,28937	34,5	407191	422099	386346	84106	56154	72025	-5,73	0,042	2,30E-05	0,044	19,8
564,35895	15,98	204618	220046	215178	0	0	0	only CTR	0	4,60E-04	0,037	0
1104,42725	28,97	87038	90916	82349	708485	703983	751313	8,31	0,712	4,20E-04	0,049	3,6
198,93996	2,184	4486	4172	2417	275710	228504	275676	70,42	28,657	3,70E-03	0,302	10,5
377,14581	15,03	15774	16069	19679	226738	222818	207782	12,76	2,198	5,00E-04	0,127	4,6
700,22876	29,88	93	0	0	372614	400100	383498	12432,39	22054,479	4,30E-04	1,732	3,6
420,16956	14,93	9394	9627	13082	379578	365909	352183	34,19	7,879	3,80E-04	0,193	3,7
329,00571	31,31	715670	670406	656739	294807	269761	295125	-2,38	0,04	3,70E-04	0,045	5,1
1104,80127	28,85	49402	49628	45398	464564	434113	505808	9,72	1,228	2,30E-03	0,049	7,7
443,29095	19,78	258538	292671	312713	0	0	0	only CTR	0	3,00E-03	0,095	0
525,67255	25,1	502	293	0	294903	278738	289684	1085,94	1065,262	2,70E-04	0,952	2,9
874,44867	22,53	626222	593115	510601	0	0	0	only CTR	0	3,50E-03	0,103	0
508,33966	35,81	106657	108560	180906	483051	482978	443339	3,56	1,314	1,10E-03	0,321	4,9
259,12915	11,56	1078	42032	3708	236929	317042	333512	18,96	31,155	4,50E-03	1,469	17,5
502,8136	19,15	2713	2151	2161	186403	248334	228291	94,38	26,447	6,90E-03	0,137	14,3
327,00861	31,41	681339	649132	649932	316607	282451	345348	-2,10	0,061	3,30E-04	0,028	10
508,33951	35,42	510957	479564	480820	9674	9722	11451	-47,70	0,003	4,40E-04	0,036	9,8
1104,42676	25,34	570548	566457	692084	0	2355	1302	-500,16	0,002	4,50E-03	0,117	96,8
1165,5946	22,69	493307	456676	384022	0	0	0	only CTR	0	5,20E-03	0,125	0
441,14804	14,92	23586	25376	35634	375814	375186	362891	13,17	3,294	5,10E-07	0,231	2
343,16559	31,43	383520	388316	391147	0	0	0	only CTR	0	3,30E-05	0,01	0
1165,9292	22,72	559375	487576	411992	0	0	282	-5173,56	0	7,60E-03	0,152	173,2
1188,87085	25,21	609320	504712	542917	0	223	1067	-1284,46	0,001	3,00E-03	0,096	130,9
464,22382	14,93	7391	6735	8752	330531	283500	256441	38,05	10,05	5,80E-03	0,135	12,9
202,04738	14,38	75120	74222	112083	223139	182055	215172	2,37	0,838	2,50E-03	0,248	10,5
387,18042	31,75	12643	12872	12232	327647	322513	323267	25,79	0,885	2,10E-05	0,026	0,9
520,32275	35,05	524440	410742	436906	198502	173854	161562	-2,57	0,092	9,50E-03	0,13	10,6
944,30792	26,04	0	0	173	247215	222203	225964	4019,55	7202,221	1,10E-03	1,732	5,8
331,05414	4,495	201091	182262	135626	570570	564766	602396	3,35	0,769	2,20E-04	0,195	3,5
1104,67627	28,96	53203	64324	56006	535588	501003	522210	8,98	1,2	1,40E-04	0,1	3,4
244,13748	14,9	70994	67585	103471	301683	281736	288246	3,60	1,01	5,10E-04	0,246	3,5
699,72797	34,32	797	250	278	505140	495254	502721	1134,43	802,634	3,30E-05	0,697	1
111,00934	2,217	1882	1231	763	277579	270184	262699	209,10	96,752	2,40E-04	0,435	2,8
1104,30151	25,26	558832	646451	649286	196	1746	370	-802,15	0,001	2,30E-03	0,083	110,1
611,95001	33,19	162	0	0	385491	414624	397546	7392,97	13083,701	4,50E-04	1,732	3,7
543,32739	31,3	343116	339741	326197	0	0	0	#DIV/0!	0	2,40E-04	0,027	0
1088,67627	35,59	15725	18097	11519	3313483	2723696	3412974	208,42	70,589	4,70E-03	0,22	11,8
1104,55188	25,43	478275	477164	553692	0	2892	1227	-366,38	0,003	2,50E-03	0,087	105,7
327,00824	31,67	593921	580156	593594	316506	277123	279930	-2,02	0,044	6,20E-04	0,013	7,5
1104,8009	27,77	2756	8713	3944	369559	516890	489231	89,25	70,018	9,70E-03	0,614	17,1
856,35712	14,9	451	392	661	226703	248323	217183	460,25	161,844	1,60E-03	0,282	6,9
591,67102	19,05	0	552	0	254024	254026	235078	1346,25	2389,461	6,40E-04	1,732	4,4
209,09203	11,96	0	889	301	234307	225060	273816	616,12	767,36	3,70E-03	1,14	10,6
510,35513	39,54	3449032	3358251	2876641	720402	529986	521600	-5,47	0,052	1,90E-03	0,095	19

373,2738	26,36	34332	849	1442	279531	236255	278445	21,69	36,063	2,20E-04	1,57	9,3
874,69897	22,52	636489	588508	514104	0	0	0	only CTR	0	3,80E-03	0,106	0
388,18396	31	3975	5294	5965	344719	363082	361758	70,21	16,013	2,50E-04	0,199	2,9
875,20001	22,71	466349	406387	347872	288	0	317	-2017,53	0,001	7,00E-03	0,146	86,9
874,69873	23,16	484913	486156	471271	0	0	0	only CTR	0	9,80E-05	0,017	0
267,13419	3,852	2876	1714	2667	223908	205148	217643	89,11	26,757	6,40E-04	0,256	4,4
874,44812	23,16	493174	466277	480271	0	1915	0	-751,81	0,002	2,40E-04	0,028	173,2
558,29541	35,2	94077	90771	94658	390332	371859	387231	4,11	0,199	2,30E-04	0,023	2,6
259,14771	2,009	5870	41135	3380	455084	397123	544564	27,72	39,276	5,80E-03	1,257	16
381,25949	29,62	232011	175394	198744	0	0	0	only CTR	0	6,50E-03	0,141	0
875,19965	24,81	130640	8647	126246	652104	521019	520787	6,38	5,843	1,30E-03	0,782	13,4
503,29605	35,01	37780	49964	47222	283837	286024	253332	6,10	1,273	7,80E-04	0,142	6,7
293,09854	19,92	159712	173692	190128	442810	500297	459198	2,68	0,403	6,30E-04	0,087	6,3
1021,62598	35,09	2124	8554	11448	278739	269281	283385	37,58	25,288	3,30E-06	0,647	2,6
569,34265	35,48	103524	86366	88031	831648	744778	852317	8,74	1,509	1,70E-03	0,102	7
1104,55164	25,21	504291	626448	682481	0	898	624	-1191,34	0,001	7,50E-03	0,151	90,7
239,96715	2,217	4116	3616	2408	226702	189585	198511	60,63	21,485	3,00E-03	0,26	9,5
1189,1571	28,79	109641	101509	89000	470134	530518	435016	4,78	0,98	4,00E-03	0,104	10,1
520,33252	15,8	160380	157282	194260	0	0	0	only CTR	0	4,80E-03	0,12	0
411,17801	20,62	495	0	0	225105	204317	212917	1297,65	2309,545	7,90E-04	1,732	4,9
293,09848	20,36	158761	167610	187588	428745	520788	531140	2,88	0,577	7,00E-03	0,086	11,4
293,0983	18,97	178512	179602	176490	510207	570841	489994	2,94	0,262	4,90E-03	0,009	8
392,19171	15,09	2215	2446	3693	219680	228696	216777	79,62	24,956	2,20E-04	0,285	2,8
431,27667	25,55	329858	276326	315804	0	0	0	only CTR	0	2,70E-03	0,09	0
508,3765	39,65	54085	105676	196236	521158	416425	439170	3,87	2,809	3,60E-03	0,606	12
160,09674	11,84	4740	14270	14455	254526	269316	329149	25,49	16,238	6,10E-03	0,498	13,9
275,14349	16,01	495	0	0	233710	208923	226182	1351,14	2417,533	1,10E-03	1,732	5,7
1041,67847	35,41	37621	39343	30410	1321352	994128	1372943	34,35	10,289	9,70E-03	0,132	16,7
1189,01282	28,86	103169	93997	73751	379776	400936	420787	4,43	0,966	5,70E-05	0,167	5,1
520,35333	35,25	78538	74027	65895	323341	275630	282806	4,04	0,708	2,90E-03	0,088	8,8
339,09058	14,15	36366	38819	38540	205917	226559	220212	5,74	0,482	9,80E-04	0,035	4,9
521,34216	36,46	525171	495340	426913	234260	216058	197594	-2,23	0,085	6,20E-03	0,104	8,5
261,0528	29,43	235736	274949	305077	1031	1114	716	-285,13	0,001	5,40E-03	0,128	22
279,13431	15,69	7082	5307	3456	190903	189652	192111	36,14	12,637	8,60E-08	0,343	0,6
512,33472	31,08	297491	299022	296675	0	354	0	-2523,13	0,001	2,90E-06	0,004	173,2
293,09839	20,54	153248	167610	187588	429204	500515	505658	2,82	0,539	2,50E-03	0,102	8,9
661,29266	14,9	3432	2329	3235	201523	181118	161237	60,46	18,578	4,20E-03	0,196	11,1
1113,6792	35,34	10874	12035	11560	287477	264564	261182	23,59	2,444	9,90E-04	0,051	5,3
413,14133	13,04	3396	9942	9965	164130	139715	139961	19,05	11,089	1,90E-03	0,487	9,5
248,09546	14,82	1497	1731	3088	281980	267985	261998	128,56	57,329	4,50E-04	0,408	3,8
432,23804	25,69	218293	250983	263808	0	0	0	only CTR	0	3,10E-03	0,096	0
1104,427	25,62	406945	431096	450742	0	4998	1582	-195,86	0,006	7,60E-04	0,051	116,5
1021,62549	34,84	283377	308913	294358	147000	145155	144669	-2,03	0,026	2,20E-03	0,043	0,8
134,99568	2,217	834	801	382	201191	161987	194904	276,69	135,022	4,30E-03	0,375	11,3

100,07542	3,082	284885	215849	240879	41878	53665	41315	-5,42	0,054	7,90E-03	0,141	15,3
525,34259	17	4204	5005	4853	0	0	0	only CTR	0	2,70E-03	0,091	0
520,32556	35,25	122264	123541	110498	304118	285514	244471	2,34	0,399	9,10E-03	0,061	11
1104,677	25,34	392714	400401	500255	1118	1892	1309	-299,46	0,001	6,40E-03	0,139	28
570,34619	35,08	301703	316745	331345	66486	67046	53993	-5,06	0,033	1,40E-04	0,047	11,8
1104,80212	28,18	50735	50767	89535	486350	469267	356618	6,87	3,522	7,10E-03	0,352	16,1
325,17615	15,09	368	424	0	184786	181044	182380	692,18	611,491	2,80E-05	0,873	1
666,229	13,91	711	1804	1328	201143	264520	237073	182,86	103,02	6,10E-03	0,428	13,6
518,32361	33,76	1959207	1897226	1768007	909146	416203	636112	-2,87	0,15	6,70E-03	0,052	37,8
790,38062	28,96	189128	198178	194502	0	0	0	only CTR	0	1,80E-04	0,023	0
547,34857	35,11	314650	301848	300840	58120	65292	52233	-5,22	0,026	2,40E-06	0,025	11,2
817,26495	33,12	0	186	0	359600	370072	382120	5977,38	10559,08	3,10E-04	1,732	3
700,22809	31,01	0	160	81	302112	324699	333382	3984,20	4170,89	8,50E-04	0,996	5
331,00241	30,74	424063	404672	427768	78656	75704	73652	-5,51	0,011	2,70E-04	0,03	3,3
188,07069	22,71	5354	0	579	215987	218055	219197	110,10	164,433	1,00E-06	1,486	0,7
503,83127	18,97	603	1246	663	162873	165486	135790	184,77	97,929	3,80E-03	0,424	10,6
160,04243	19,77	165	0	0	214807	256706	279779	4553,28	8511,969	5,70E-03	1,732	13,2
1104,05042	28,15	11109	42202	72451	367664	313072	308666	7,87	6,54	3,90E-04	0,732	10
566,32129	35,64	111034	109998	104473	606687	580475	711820	5,83	0,83	5,70E-03	0,033	11
583,1908	29,97	0	0	208	502709	498300	493348	7184,41	12531,812	2,90E-05	1,732	0,9
478,29236	31,78	262022	279023	232407	0	0	0	only CTR	0	2,80E-03	0,092	0
875,45093	24,63	22786	13786	18726	417539	563439	488969	26,58	10,458	7,70E-03	0,245	14,9
1104,30188	25,62	380966	390914	429241	521	4301	1096	-202,96	0,005	1,30E-03	0,064	103,3
197,07849	17,65	7167	0	331	220092	240337	261600	96,30	164,152	1,80E-03	1,619	8,6
520,36206	35,24	116401	105688	88420	284050	273615	257015	2,62	0,49	1,20E-04	0,136	5
525,34296	19,21	680	1477	467	196379	174241	179305	209,58	140,842	1,30E-03	0,609	6,3
1188,87024	28,78	89439	87927	76593	389582	417351	350596	4,56	0,774	3,00E-03	0,083	8,7
468,30881	30,95	672458	790784	674749	0	0	0	only CTR	0	3,00E-03	0,095	0
998,62946	34,73	257537	237857	207015	62123	53216	47917	-4,30	0,056	4,10E-03	0,109	13,2
1040,51343	25,41	407062	311192	336483	0	0	3283	-321,27	0,006	6,60E-03	0,141	173,2
586,35828	28,44	0	3427	1889	223630	209358	236271	125,90	129,54	1,10E-03	0,969	6
132,10733	3,923	881385	1562074	1563401	2914939	3058706	2598977	2,14	0,806	8,20E-03	0,295	8,2
1189,29956	28,89	79223	70912	69852	317517	379350	319489	4,62	0,803	5,00E-03	0,07	10,4
582,29462	35,41	61296	58215	49029	330172	344668	360870	6,15	0,971	1,90E-04	0,114	4,4
327,07843	38,78	396106	386576	356522	59352	38215	46523	-7,91	0,035	1,50E-04	0,054	22,2
465,30386	19,79	224944	182927	259469	0	0	0	only CTR	0	9,80E-03	0,172	0
1040,76392	28,18	118677	94945	127471	325643	393920	424255	3,35	0,94	6,80E-03	0,148	13,2
374,06323	32,74	889490	889941	934374	316944	311358	366320	-2,73	0,044	1,90E-05	0,029	9,1
500,30405	22,23	20054	19268	19097	126285	140142	157522	7,26	0,994	5,40E-03	0,026	11,1
381,25964	30,52	295791	308941	309407	0	0	0	only CTR	0	2,10E-04	0,025	0
293,09811	20,1	159712	172807	192744	411329	488340	533133	2,73	0,611	9,60E-03	0,095	12,9
1189,1571	25,62	447420	402457	399789	1086	4196	5742	-113,36	0,006	1,30E-03	0,064	64,5
311,16034	11,76	0	1494	1746	180660	188453	197954	175,02	160,935	6,20E-04	0,874	4,6
362,08484	15,28	31313	31551	16364	159052	167583	190893	6,53	2,776	8,10E-04	0,329	9,6

1165,59558	24,84	73456	6763	79084	406562	445913	392940	7,82	6,441	4,30E-04	0,758	6,6
372,0658	32,77	985130	985188	1030734	351940	352016	384780	-2,76	0,028	1,10E-05	0,026	5,2
678,31909	14,85	1847	2241	7768	149406	136150	141424	36,01	31,861	7,90E-05	0,838	4,7
1049,33923	30,4	0	243	0	407993	420211	413208	5108,69	8930,349	7,30E-05	1,732	1,5
394,22601	22,43	275810	286407	275303	0	0	0	only CTR	0	1,70E-04	0,022	0
369,15857	15,27	0	0	383	199162	214965	223838	1665,70	2980,658	1,10E-03	1,732	5,9
1166,26257	22,69	347706	332503	247854	0	0	0	only CTR	0	9,90E-03	0,174	0
413,27988	22,91	154	0	0	146948	179369	198672	3409,02	6421,669	7,40E-03	1,732	14,9
1189,01416	25,61	449934	395404	354978	983	3688	5022	-123,83	0,006	4,70E-03	0,119	63,7
699,72803	34,12	797	2138	623	477016	419202	392300	362,15	289,73	3,40E-03	0,699	10,1
402,15918	14,92	3541	4679	4782	236424	206376	218573	50,87	11,574	1,60E-03	0,159	6,9
1104,17676	25,38	376408	387599	457115	324	0	818	-1069,28	0,001	3,80E-03	0,107	108,2
243,13402	24,79	449453	443333	468086	200340	225481	227097	-2,08	0,047	3,90E-05	0,028	6,9
293,09845	21,88	155701	159838	185305	323060	399542	385799	2,21	0,457	6,70E-03	0,096	11
1040,51294	25,17	443583	374865	414647	0	1610	1380	-412,41	0,002	2,30E-03	0,084	87,4
1104,30078	25,44	461026	511803	531065	521	3518	0	-372,34	0,004	1,70E-03	0,072	141
192,06561	14,15	9224	8638	10828	245873	231844	223765	24,45	4,069	7,40E-04	0,119	4,8
143,10652	17,04	67788	72618	68060	227651	249311	221334	3,35	0,342	2,00E-03	0,039	6,3
1165,92981	24,87	71563	10081	86185	420764	367186	336735	6,70	5,598	7,20E-04	0,722	11,3
293,09821	22,06	150020	158503	175206	408348	391747	382289	2,44	0,276	2,60E-05	0,079	3,3
1189,29932	25,5	484452	399936	417463	510	4058	3352	-164,38	0,005	3,50E-03	0,103	71,2
1104,55225	25,66	346561	368661	388250	0	6407	4913	-97,48	0,01	8,50E-04	0,057	88,8
164,0706	12,93	217731	190894	220003	50454	61412	50599	-3,87	0,05	1,30E-03	0,077	11,6
372,06631	31,16	485147	449301	504686	62963	59728	60149	-7,87	0,011	1,40E-03	0,059	2,9
1104,04993	27,89	3216	6504	10904	348204	397005	319156	51,61	34,678	3,90E-03	0,561	11,1
547,34778	35,38	33657	38172	33996	288248	262542	301717	8,06	1,138	1,90E-03	0,071	7
409,16226	28,96	292257	294677	264905	0	0	0	only CTR	0	1,10E-03	0,058	0
1040,6377	25,24	446217	382476	382769	0	503	560	-1139,66	0,001	2,70E-03	0,091	87
543,32452	35,25	136628	146324	127039	313581	273274	285610	2,13	0,301	1,70E-03	0,071	7,1
374,06335	31,16	458934	429499	495428	60303	54481	54439	-8,18	0,016	2,00E-03	0,072	6
132,10693	3,747	673091	615028	633002	1336864	1463898	1493309	2,24	0,234	1,50E-03	0,046	5,8
331,2269	32,6	36567	36143	32091	271457	253757	264912	7,54	0,79	2,20E-04	0,071	3,4
1104,427	29,18	72939	74554	65725	406257	411345	389677	5,66	0,534	5,80E-05	0,066	2,8
1078,92871	29,04	0	0	76	220500	230144	230036	8956,32	15681,584	2,00E-04	1,732	2,4
335,22278	17,24	3114	2502	2682	134962	156931	155806	53,95	10,609	2,40E-03	0,114	8,3
524,67175	34,34	219	0	0	484961	457079	454850	6378,49	11301,896	4,30E-04	1,732	3,6
181,06079	12,4	335	1618	1067	241851	223098	222913	227,77	156,556	7,30E-04	0,64	4,7
481,30264	18,97	1573	1936	1132	142692	155857	130366	92,42	32,266	2,70E-03	0,26	8,9
215,09305	2,009	13009	72238	7626	391400	456498	447068	13,94	17,284	1,60E-04	1,158	8,1
1040,76379	25,26	358770	318426	318210	0	1332	1626	-336,51	0,003	1,60E-03	0,07	87,9
543,3244	34,98	296703	313891	309672	141386	141574	105835	-2,37	0,079	1,40E-03	0,029	15,9
544,35938	35,41	29193	36616	27970	297387	320678	340720	10,22	2,224	1,30E-03	0,15	6,8
508,37677	39,98	382805	421958	416156	148965	91930	96188	-3,62	0,092	4,10E-04	0,052	28,3
134,10576	3,737	114802	118689	107186	250152	228244	222834	2,06	0,233	1,70E-03	0,052	6,2

368,15533	15,09	917	2799	2367	649513	631677	776635	338,29	203,438	4,40E-03	0,486	11,5
523,34851	35,05	315337	312864	296065	123183	126626	107599	-2,59	0,046	2,40E-05	0,034	8,5
392,27985	26,7	51476	358	292	198153	199311	199695	11,46	19,518	8,70E-03	1,7	0,4
148,07552	20,09	497	193	0	190910	177828	178413	792,97	896,575	5,40E-04	1,09	4,1
190,04973	13,35	1247	0	0	602383	643569	527733	1422,36	2604,359	3,30E-03	1,732	9,9
299,20081	29,52	222178	225636	233987	0	0	240	-2840,84	0,001	2,40E-04	0,027	173,2
874,94989	22,53	454889	428807	355261	0	0	0	only CTR	0	5,20E-03	0,125	0
293,09821	23,47	119100	128707	140772	356996	417390	450223	3,15	0,629	7,10E-03	0,084	11,6
482,32407	31,68	255591	291051	306152	0	0	0	only CTR	0	2,80E-03	0,091	0
818,58679	25,52	146517	160284	167583	0	0	0	only CTR	0	1,50E-03	0,068	0
293,09821	23,76	98422	120007	130505	367528	396176	401115	3,34	0,625	4,60E-05	0,141	4,7
243,11313	16,16	0	360	0	138804	157618	154706	1253,13	2255,313	1,50E-03	1,732	6,7
699,56238	32,26	208	0	0	319238	286105	298114	4343,54	7771,868	1,00E-03	1,732	5,6
1104,05017	28,89	37526	39516	43305	323972	351300	377784	8,75	1,311	2,30E-03	0,073	7,7
457,12192	14,92	5661	5465	7919	239114	237662	235484	37,40	8,323	2,20E-08	0,215	0,8
129,02	2,217	3711	2258	1528	195860	198092	194289	78,46	35,652	2,60E-07	0,445	1
130,01677	2,217	3436	2180	1317	172505	189440	192270	79,94	41,497	1,00E-03	0,461	5,8
496,33014	31,57	284184	266755	275563	0	0	0	#DIV/0!	0	3,30E-04	0,032	0
450,17184	13,64	6386	4711	4222	251143	231459	237141	46,98	12,429	5,40E-04	0,222	4,2
1050,33923	32,42	0	158	0	383458	341087	369515	6924,43	12380,542	1,20E-03	1,732	5,9
836,61591	25,53	173558	179402	180495	0	0	0	only CTR	0	1,50E-04	0,021	0
544,35883	35,1	330785	290568	379039	43727	64044	58103	-6,03	0,053	6,10E-03	0,133	18,9
240,12331	12,61	392	389	735	192590	164010	187478	358,89	171,301	2,40E-03	0,393	8,4
269,15768	29,42	267824	255699	300791	1189	1210	1645	-203,84	0,001	2,40E-03	0,085	19,1
133,09857	3,882	103443	127806	154094	313947	253548	332496	2,34	0,782	6,40E-03	0,197	13,8
465,30444	23,87	0	118	0	170844	191608	169916	4511,59	8119,609	1,60E-03	1,732	6,9
874,94928	23,16	328904	319893	346578	0	0	0	only CTR	0	5,60E-04	0,041	0
1104,30029	29,14	71437	72809	70907	409175	362000	380852	5,35	0,404	1,90E-03	0,014	6,2
1189,44263	25,17	460833	365415	427330	0	0	816	-1536,25	0,001	4,40E-03	0,116	173,2
293,09796	25,1	113950	128895	168949	313968	315432	397869	2,49	0,867	6,10E-03	0,207	14
520,36169	35,05	420567	426079	329781	156445	137172	106525	-2,94	0,111	6,00E-03	0,138	18,9
257,11343	15,77	162339	132591	137467	46607	51997	62216	-2,69	0,096	3,40E-03	0,111	14,8
438,29764	37,68	63683	73045	75649	275677	272953	240820	3,72	0,604	1,60E-03	0,089	7,4
293,09821	26,16	104649	153747	107435	289994	393663	355920	2,84	1,073	7,00E-03	0,226	15,1
1104,92639	27,89	4894	4614	7378	251849	308730	238787	47,34	19,406	6,60E-03	0,27	14
293,09845	25,28	108062	136741	122321	329350	383544	372038	2,96	0,58	1,10E-03	0,117	7,9
1104,67737	25,53	339354	319489	368030	494	1043	1346	-356,18	0,001	1,70E-03	0,071	44,9
1104,42712	29,36	53399	63296	59807	369820	313521	291187	5,52	1,16	7,00E-03	0,085	12,5
345,18436	3,786	63309	83399	105789	241342	177931	204952	2,47	1,002	7,40E-03	0,252	15,3
544,31604	35,1	282655	319133	307725	50310	57867	56859	-5,51	0,025	1,20E-03	0,062	7,5
172,04022	3,179	56194	58928	57540	18825	15530	4032	-4,50	0,14	8,30E-03	0,024	60,7
524,67169	34,52	219	0	0	416084	374338	406045	5463,32	9782,167	9,90E-04	1,732	5,5
1040,38782	25,21	356025	293540	330917	0	0	303	-3235,91	0,001	3,10E-03	0,096	173,2
230,15833	4,398	8715	13252	6260	281381	251930	266976	28,35	12,254	6,20E-04	0,377	5,5

1104,67639	29,14	40329	57305	51465	333052	316418	291832	6,31	1,513	5,20E-04	0,174	6,6
391,26703	22,83	574	522	442	190365	183808	185198	363,70	53,652	1,10E-04	0,129	1,9
404,15515	15,27	86027	87323	52732	144504	153459	163050	2,04	0,654	9,60E-03	0,26	6
252,12679	19,52	0	0	6755	130920	158854	132424	62,50	115,236	2,80E-03	1,732	11,2
293,09827	25,51	91908	103906	91249	294594	363908	349616	3,51	0,644	6,10E-03	0,074	10,9
1015,67139	36,24	290744	262811	237238	134739	97513	100088	-2,38	0,122	1,80E-03	0,102	18,8
157,08586	15,25	10360	10087	10671	185923	151130	177561	16,54	2,218	4,20E-03	0,028	10,6
160,04247	19,3	166	337	0	216395	226737	266527	1410,85	1572,499	4,20E-03	1,005	11,2
293,09793	25,93	117576	171324	125403	315229	377975	381851	2,59	0,816	1,70E-03	0,21	10,4
699,56122	30,38	208	0	0	279889	279022	285611	4060,20	7090,222	5,30E-05	1,732	1,3
143,10648	16,75	79188	75994	73606	190407	176638	213187	2,54	0,335	7,10E-03	0,037	9,5
382,27176	31,06	234842	234489	232527	0	0	0	only CTR	0	9,50E-06	0,005	0
567,32465	35,18	300631	313215	300974	99114	104535	82646	-3,20	0,045	4,70E-05	0,024	11,9
523,34802	35,24	72686	76160	76167	247440	248440	258744	3,35	0,173	1,30E-04	0,027	2,5
509,32962	23,55	185	0	0	174042	170754	171860	2792,74	4861,159	2,90E-05	1,732	1
1188,87012	25,68	301370	312688	273304	0	2497	5316	-113,58	0,01	1,40E-03	0,069	102,1
1188,72729	25,17	382005	286287	362404	0	1607	1899	-293,98	0,003	7,20E-03	0,147	87,5
699,729	33,37	3029	2851	2961	275383	283341	259147	92,51	7,005	7,00E-04	0,03	4,5
481,30258	17,01	2803	2444	2804	0	0	0	only CTR	0	2,00E-03	0,077	0
227,07918	12,05	215674	295960	242379	40105	54826	61981	-4,81	0,078	9,70E-03	0,163	21,3
185,11725	17,7	24802	16797	12569	204975	190649	148487	10,04	5,083	8,30E-03	0,344	16,2
435,17642	19,74	2960	4696	3182	151199	169525	139839	42,50	15,262	3,20E-03	0,262	9,8
875,19952	22,9	353119	345566	312589	0	0	159	-6360,21	0	1,40E-03	0,064	173,2
567,32446	35,48	71380	64977	66931	268892	264238	280262	4,00	0,315	1,00E-04	0,048	3
231,11635	2,009	17656	69267	13025	293626	345719	342397	9,82	10,08	2,90E-04	0,937	8,9
817,36591	12,1	143562	136163	131222	0	5791	6876	-32,44	0,028	3,50E-05	0,045	87,6
404,20737	29,41	65269	71742	80249	161315	177770	174138	2,36	0,364	1,30E-04	0,104	5,1
1040,63721	25,5	322571	302345	302497	0	1708	1431	-295,45	0,003	4,40E-04	0,038	87,6
279,15933	2,009	14892	87715	11280	305807	383675	364783	9,26	11,586	8,00E-04	1,136	11,6
327,0083	2,009	9289	56764	6180	289126	311508	323537	12,79	15,79	3,80E-04	1,177	5,7
192,03255	11,82	3623	32881	17244	220389	228929	186143	11,82	10,925	5,70E-04	0,817	10,7
1104,17639	25,65	236408	284289	317158	673	3283	1128	-164,80	0,006	7,00E-03	0,145	82,3
392,28802	29,09	76572	77019	87456	221116	197271	212839	2,62	0,351	5,10E-04	0,077	5,8
411,32986	34,46	229937	196987	199474	24163	19853	17297	-10,22	0,025	2,40E-03	0,088	17
331,00247	30,93	356260	360812	340921	75252	79883	82548	-4,45	0,017	1,20E-04	0,03	4,7
1050,34192	32,23	0	158	0	344361	348124	323077	6427,61	11368,308	5,30E-04	1,732	4
425,1532	20,26	2057	1323	1085	198322	193226	197311	131,88	46,726	3,70E-05	0,341	1,4
376,06012	30,74	291675	265946	312133	20239	16721	17033	-16,11	0,012	2,30E-03	0,08	10,8
372,06592	31,35	366468	360791	350728	72175	60303	71610	-5,28	0,023	1,50E-06	0,022	9,8
526,29181	35,06	1213	9105	4288	219664	210865	204939	43,51	37,067	2,40E-05	0,817	3,5
570,35614	37,64	487625	629981	672605	320229	190952	246604	-2,36	0,177	9,90E-03	0,162	25,7
350,02243	14,74	201	0	0	317370	299498	381728	4968,14	9256,163	5,60E-03	1,732	13
1015,67175	36,65	247067	212049	200126	118397	100254	90704	-2,13	0,116	4,50E-03	0,111	13,6
987,64142	31,59	177831	186043	163505	0	0	0	only CTR	0	1,40E-03	0,065	0

241,1548	3,51	19626	16022	20038	173026	153673	133531	8,26	2,048	6,50E-03	0,119	12,9
517,33044	24,96	18465	0	23354	137708	126192	159650	10,13	10,169	7,40E-04	0,884	12
287,63303	34,98	192896	194259	173471	86426	89313	73886	-2,25	0,072	4,20E-04	0,062	9,9
1040,51306	25,59	294501	234982	246737	0	0	4214	-184,20	0,01	4,80E-03	0,122	173,2
520,31854	35,25	95048	101041	86245	277493	315006	360300	3,37	0,707	9,50E-03	0,079	13,1
242,1217	16,62	168	0	0	117955	140491	140365	2373,88	4337,022	3,20E-03	1,732	9,8
1104,17578	29,12	53283	58422	52750	339927	312532	291381	5,74	0,772	2,60E-03	0,057	7,7
468,30841	29,55	170755	176733	193751	338	346	0	-791,29	0,001	1,50E-03	0,066	86,6
374,0629	31,35	377253	371216	342735	74232	59476	64313	-5,51	0,03	2,90E-04	0,051	11,4
317,1427	19,53	0	0	26405	138375	142391	131788	15,62	27,67	2,10E-03	1,732	3,9
424,19232	3,967	7994	39229	52032	146711	127268	163561	4,41	3,567	3,00E-03	0,685	12,5
1104,30042	29,39	42614	51624	60965	328375	298424	262063	5,73	1,658	3,80E-03	0,177	11,2
171,14909	21,19	5494	5614	5492	223612	209508	216838	39,15	1,767	3,70E-04	0,013	3,3
1022,62927	35,09	1486	747	3009	156966	136379	156515	85,82	63,395	1,90E-03	0,66	7,8
419,16602	14,75	1018	1656	4816	925620	1159463	1092857	424,29	393,847	4,30E-03	0,815	11,4
276,15561	11,56	0	15320	981	105152	148442	140072	24,15	42,34	5,50E-03	1,578	17,5
411,1698	13,9	1421	1730	385	153726	125634	116425	111,93	83,376	7,20E-03	0,598	14,7
293,09811	27,06	117455	128707	133072	251128	280769	252271	2,07	0,265	1,30E-03	0,064	6,4
311,12573	19,2	0	549	0	150145	140754	137371	780,09	1387,611	7,00E-04	1,732	4,6
329,0054	2,097	7256	8727	3684	221982	252721	270993	37,92	18,777	3,20E-03	0,396	10
424,30606	23,57	5131	5214	6415	193	175	281	-25,82	0,015	5,70E-03	0,129	26,2
1104,55115	29,32	57447	50307	59484	332138	278098	312078	5,51	0,967	3,10E-03	0,086	8,9
288,1922	22,36	137928	103553	121238	0	370	898	-286,06	0,004	6,70E-03	0,142	106,7
218,02136	14,38	13156	11178	18219	100520	94050	90233	6,69	2,08	6,00E-05	0,256	5,5
432,28033	15,18	26931	32189	48560	116284	113034	95238	3,01	1,263	1,40E-03	0,314	10,5
293,09827	27,29	109498	119825	126631	243636	217948	265054	2,04	0,347	6,30E-03	0,073	9,7
354,28485	35,33	221619	207018	200528	0	670	1653	-270,84	0,004	8,40E-04	0,052	107,4
425,13583	30,94	519	949	1918	176085	194312	181544	163,01	111,832	8,20E-04	0,635	5,1
1104,42615	25,79	359524	320032	333801	1580	9121	3044	-73,73	0,013	8,20E-04	0,059	87,3
503,31525	19,15	0	1543	983	89478	113809	95037	118,10	124,725	5,40E-03	0,928	12,8
227,07909	12,25	263322	242070	267707	68988	93930	98130	-2,96	0,079	1,60E-04	0,053	18,1
611,94867	32,99	162	0	0	298192	294850	303237	5532,59	9666,535	6,60E-05	1,732	1,4
348,21732	31,81	20763	32138	34089	161816	151082	154366	5,37	1,523	3,00E-05	0,248	3,5
1189,15759	28,97	85205	73976	69431	323819	304242	271079	3,93	0,769	2,50E-03	0,107	8,9
1040,76379	25,44	302461	256371	257452	0	2392	871	-250,16	0,005	3,10E-03	0,097	111,3
874,19763	23,13	244046	282475	266404	0	0	532	-1490,46	0,001	1,80E-03	0,073	173,2
175,0965	15,25	5287	6651	7807	148759	135927	148938	21,96	5,344	7,30E-04	0,192	5,2
289,15106	3,638	5374	8978	10353	96950	90072	79624	10,79	4,43	2,10E-03	0,312	9,8
1061,65454	35	145320	158221	156130	69488	65425	45143	-2,55	0,103	1,50E-03	0,045	21,7
700,22809	30,08	193	0	0	377501	320592	275839	5046,28	9557,082	8,10E-03	1,732	15,7
1189,58667	25,21	311094	246268	277986	0	582	922	-555,42	0,002	4,50E-03	0,116	93
1048,33691	33,99	187	0	0	310020	334853	309655	5104,43	9057,123	6,90E-04	1,732	4,5
243,12308	17,29	509	176	0	150163	165284	148550	677,37	808,413	1,20E-03	1,133	6
420,19791	20,44	498	802	496	689750	673740	670269	1132,38	349,968	7,80E-05	0,294	1,5

293,09808	26,36	94611	146126	124188	277013	270344	318429	2,37	0,718	1,40E-03	0,213	9
520,33984	38,25	290133	268563	253464	133678	146841	115725	-2,05	0,091	6,60E-04	0,068	11,8
469,31192	32,48	21678	17328	33084	553167	556398	551782	23,05	7,902	2,20E-05	0,339	0,4
293,09824	28,15	104248	146951	120439	232555	244887	301732	2,10	0,663	9,70E-03	0,174	14,2
192,12315	17,47	18385	850	1072	211155	177403	203488	29,15	45,951	3,70E-04	1,486	9
311,1239	8,715	10332	28792	25943	232108	180057	211534	9,59	5,601	2,80E-03	0,458	12,6
372,0657	31,54	300558	316745	292198	78087	66024	73024	-4,19	0,03	1,20E-04	0,041	8,4
1043,68457	35,05	141042	137827	125132	49047	46701	37203	-3,04	0,067	1,90E-04	0,062	14,2
1067,13245	34,99	100119	108564	88546	9252	13534	14973	-7,87	0,043	2,50E-03	0,101	23,6
1189,58557	25,44	234002	176261	209572	0	2431	865	-188,06	0,007	6,50E-03	0,14	112,1
1067,68433	35,09	157686	150925	146284	14737	17733	20317	-8,62	0,023	6,00E-05	0,038	15,9
1104,80139	25,44	240517	245136	269555	0	0	691	-1092,92	0,002	1,30E-03	0,062	173,2
875,4505	24,84	55521	3573	66800	185976	219519	190246	4,73	4,238	5,30E-03	0,804	9,2
1040,88916	25,31	268700	213049	234185	0	450	619	-669,72	0,002	4,60E-03	0,118	89,8
1022,62823	34,83	159136	163090	163912	83101	73969	80671	-2,04	0,037	9,70E-05	0,016	6
1040,38733	25,44	277730	232102	267548	473	1796	1318	-216,72	0,003	2,80E-03	0,092	56
699,72821	33,65	1818	2455	1692	278379	298357	277477	143,20	35,412	5,70E-04	0,206	4,1
320,07687	15,2	373	0	0	112832	107180	104825	870,88	1543,354	4,70E-04	1,732	3,8

B: NEGATIVE ION

		pool rat plasma control (CTR)			pool rat plasma 2h postresuscitation after cardiac arrest (CPR2)			Fold change			CV (%)	CV (%)
MZ	**RT**	**Replicate 1**	**Replicate 2**	**Replicate 3**	**Replicate 1**	**Replicate 2**	**Replicate 3**	**CPR2 vs CTR**	**SD**	**p Value**	**pool CTR**	**pool CPR2**
348,10306	11,81	2427	262	1946	27158746	24033276	23354333	16083,36	13143,97	2,20E-003	73,6	8,2
241,11969	15,51	0	347400	263395	16741165	18007758	19222338	88,36	84,759	1,30E-003	89	6,9
322,12354	4,623	469	0	4635	8634990	8824199	8886613	5161,80	7819,006	7,40E-005	150	1,5
313,06549	2,356	4334971	3859101	3888211	10763680	10595456	10032767	2,60	0,267	4,50E-005	6,6	3,7
697,21295	15,45	0	1882	0	9164013	9238368	9607148	14882,85	26161,16	2,20E-004	173,2	2,5
241,11975	14,66	0	488304	334	9153942	9435939	8098817	54,62	98,833	6,20E-004	173	7,9
348,10263	14,9	0	988	0	8449280	8779152	7712894	25244,26	45384,32	1,40E-003	173,2	6,6
164,07199	5,742	4203177	4033509	3959711	14087	6725	6572	-445,38	0,001	3,10E-004	3,1	47,1
697,71454	15,45	0	1356	0	6741608	7086724	7145230	15467,23	27282,38	3,20E-004	173,2	3,1
239,104	16,35	25026	3809	27436	7093588	7474004	6774197	379,27	281,573	8,00E-004	69,3	4,9
178,05125	4,615	1005465	674814	2248	4867470	5251560	5223113	9,12	8,693	1,30E-003	91,2	4,2
164,07204	5,537	4203177	4174634	4519886	37247	20341	32169	-143,70	0,002	6,60E-004	4,5	29
514,28516	20	593931	608121	617501	4884802	5023818	4634039	7,99	0,482	6,90E-004	2	4,1
277,2178	46,29	7699107	7389022	7279582	1090572	809929	840673	-8,16	0,024	5,40E-006	2,9	16,8
329,03958	2,339	422160	430753	419021	3804789	3949719	3578507	8,91	0,569	1,00E-003	1,4	5
164,07204	4,976	0	141125	394	3308743	4252836	3698532	79,57	147,295	4,40E-003	172,5	12,6
697,21332	14,88	0	8009	0	4192444	3955613	5027518	1645,10	3060,273	5,40E-003	173,2	12,8
512,26959	18,54	623830	643124	615093	3498316	3733202	3539101	5,72	0,331	5,20E-004	2,3	3,5
522,6582	15,4	3243	0	346	3198868	3059070	2768687	2515,08	3927,197	1,80E-003	148,9	7,3
349,10596	11,81	1730	0	1363	4705603	4113454	3978374	4137,55	4032,711	2,70E-003	88,4	9,1
464,47299	15,45	0	415	0	3604618	3623332	3515927	25888,86	45274,16	8,60E-005	173,2	1,6

129,05615	4,535	744368	787959	1079594	2251172	2929965	2343975	2,88	1,026	6,70E-003	20,9	14,7
172,99152	5,669	4392	622036	0	4354152	5135921	3976421	21,50	39,676	1,10E-003	171,4	13,2
227,20198	47,27	4728938	5313641	3918454	926953	924790	683411	-5,51	0,057	9,00E-003	15,1	16,6
322,12354	3,582	0	0	2625	2401861	2749553	2508387	2918,02	5257,493	1,60E-003	173,2	7
253,21773	48,3	3893758	3714815	4607091	1287676	1169916	1062534	-3,47	0,061	6,30E-003	11,6	9,6
523,15973	15,4	0	0	352	1900395	1738100	1558230	14763,42	27066,83	3,20E-003	173,2	9,9
351,02142	2,356	404740	380363	364986	2040055	2029906	1847133	5,14	0,552	1,10E-003	5,2	5,5
401,15707	14,46	0	80691	0	2906929	2808816	3163026	110,03	197,385	7,00E-004	173,2	6,2
201,02289	15,36	3311	201709	3154	1850057	2190849	2586363	31,84	57,882	5,80E-003	165,1	16,7
697,2135	16	10156	1848	6321	2164445	2194064	1986769	346,26	254,076	9,30E-004	68,1	5,3
327,23343	47,27	2820524	3199440	2547276	1241015	966784	637448	-3,01	0,144	1,80E-003	11,5	31,9
167,02132	2,816	125406	54416	175087	2028888	2208924	2082247	17,81	9,911	2,40E-005	51,3	4,4
697,71527	15,07	0	5562	0	1422041	1904827	1611011	887,79	1669,049	7,20E-003	173,2	14,8
269,08813	2,663	396244	434117	466115	1767957	1614978	1436193	3,72	0,685	5,00E-003	8,1	10,3
242,12302	15,52	908	41504	33425	2278785	2147867	2387244	89,85	81,121	6,80E-004	85	5,3
698,21649	14,88	0	693	0	1487950	1399271	1722629	6652,02	12244,75	3,90E-003	173,2	10,9
255,09882	6,152	994632	20113	909265	3071715	2118025	3059136	4,29	4,461	9,00E-003	84,2	19,9
255,08052	14,34	0	5133	0	1377754	1616766	1494968	874,63	1584,827	2,10E-003	173,2	8
291,08371	2,789	729300	809993	623921	2432682	2410769	2444445	3,37	0,46	7,10E-004	12,9	0,7
483,24713	15,52	0	1356	1773	1514409	1414730	1471454	1406,39	1297,66	3,90E-004	88,9	3,4
515,28845	20	150084	162910	156810	1366785	1417905	1313606	8,72	0,69	5,20E-004	4,1	3,8
339,20016	31,81	2943396	2537092	2406540	645573	597449	646230	-4,17	0,036	6,10E-003	10,6	4,4
228,00998	13,2	1584	1431	2256	1089731	1024345	1055673	601,36	168,892	3,20E-004	25	3,1
314,06879	2,356	433940	419291	403764	1189735	1133261	1108049	2,73	0,198	3,30E-004	3,6	3,7
698,21515	16	2427	0	890	1202226	1182036	1044689	1033,75	1225,719	1,90E-003	111	7,5
225,12471	24,99	3794252	2920729	3063161	1320848	963711	939853	-3,03	0,113	6,60E-003	14,4	19,9
239,10393	16,86	1373986	1475114	1402625	90630	91249	45474	-18,70	0,02	4,00E-005	3,7	34,6
579,3913	40,83	2497586	2689616	2324720	1336645	1099498	995311	-2,19	0,103	7,40E-004	7,3	15,3
115,04044	3,492	72936	35307	39368	719872	612784	655272	13,47	6,749	8,40E-004	42	8,1
325,18463	30,43	2324614	1794859	2126628	613552	1010188	907524	-2,47	0,151	3,90E-003	12,9	24,4
225,11957	24,47	1352863	1431632	1128948	595743	506947	337760	-2,72	0,145	2,50E-003	12	27,3
357,10797	11,23	0	2424	0	899127	1206095	1157623	1346,06	2535,566	7,60E-003	173,2	15,2
157,03693	2,737	119470	102514	95906	1407628	1499676	1140487	12,73	3,221	7,20E-003	11,5	13,8
513,27289	18,54	169475	167204	163150	978054	1045003	980687	6,01	0,343	6,40E-004	1,9	3,8
465,2865	21,8	400198	422828	412650	1179163	1402636	1209902	3,07	0,379	6,30E-003	2,8	9,6
329,03058	2,285	1187242	1289008	1363950	328751	170613	257370	-5,07	0,076	1,30E-004	6,9	31,4
311,16882	28,7	1695570	2237839	1727199	502330	760044	793990	-2,75	0,143	8,90E-003	16,1	23,3
464,47348	15,07	0	895	0	791774	975426	768605	2833,30	5287,811	5,90E-003	173,2	13,4
582,37903	41,69	1940236	1784401	2233864	1129277	913395	843342	-2,06	0,131	4,80E-003	11,5	15,5
473,23862	24,47	1711149	1331660	1387793	624127	474239	445699	-2,87	0,113	6,20E-003	13,9	18,6
329,15109	5,537	667679	627244	524120	0	0	0	only CTR	0	4,90E-003	12,2	0
172,99158	6,26	872696	748432	925511	63527	99873	76430	-10,62	0,032	3,50E-003	10,7	23
514,28571	18,21	346502	297910	367035	1316088	1308451	1175986	3,76	0,629	5,30E-004	10,5	6,2
278,22098	46,29	1505933	1415395	1423960	203831	152759	163590	-8,35	0,023	3,10E-005	3,5	15,5

253,21764	48,51	1428601	1736879	1515380	555505	528700	452294	-3,05	0,068	4,00E-003	10,2	10,5
254,98195	2,789	4806	6386	0	556675	541093	600276	151,72	143,482	8,80E-004	89,2	5,4
242,12299	14,66	0	56730	0	1104096	1141911	980826	56,88	102,974	6,30E-004	173,2	7,8
498,29041	19,92	119566	141337	139963	879648	881870	785647	6,35	0,991	1,30E-003	9,1	6,5
172,99146	6,077	679663	933402	864710	451102	363311	245694	-2,34	0,193	9,20E-003	15,9	29,2
579,39136	40,47	1767576	2359442	2302715	718751	524771	442531	-3,81	0,106	6,30E-003	15,2	25,2
464,80777	15,07	0	287	0	598167	761564	575478	6742,89	12725,15	8,20E-003	173,2	15,7
580,39459	40,71	1214653	1222425	1076972	660156	527910	477650	-2,11	0,114	1,10E-003	7	17
522,65851	15,07	0	0	346	638139	838286	735580	6393,08	11936,49	6,10E-003	173,2	13,6
329,03055	2,081	1200614	1199307	1240318	401761	373567	398753	-3,10	0,019	4,00E-006	1,9	4
262,01382	5,207	321190	376270	458682	0	40943	12651	-21,57	0,063	7,40E-003	18	117,3
269,06686	3,881	0	318	0	634012	741394	708411	6552,88	11885,02	2,10E-003	173,2	7,9
273,20746	47,27	1078083	1161718	872445	198585	193653	154345	-5,69	0,049	8,60E-003	14,3	13,3
465,3053	40,4	1567404	1222594	1258660	306882	254948	194541	-5,35	0,068	6,10E-003	14	22,3
283,10388	2,712	125146	69836	86254	636926	772388	573137	7,05	3,221	7,00E-003	30,3	15,4
165,07516	5,742	413889	410262	407266	1625	0	861	-495,34	0,002	7,60E-006	0,8	98,1
301,21774	45,33	909283	870981	836095	277486	203997	206711	-3,80	0,059	4,10E-005	4,2	18,2
405,26511	21,8	211508	221632	229805	651421	781723	678464	3,19	0,443	6,00E-003	4,1	9,8
609,51105	49,36	887172	862733	759771	171053	197685	180990	-4,57	0,034	2,60E-003	8,1	7,3
483,24698	14,66	0	7433	0	591976	531251	459517	212,93	395,581	5,10E-003	173,2	12,6
357,10178	14,8	7115	0	275	489128	448368	461842	189,36	318,247	4,40E-004	163,6	4,5
299,22348	48,3	891606	821293	1005749	262917	248816	238576	-3,62	0,042	6,00E-003	10,3	4,9
179,05449	4,615	142442	145590	90806	475306	572254	570025	4,27	1,478	1,20E-003	24,4	10,3
173,08203	3,728	2657	4948	1580	743568	778611	768867	249,43	146,056	1,70E-004	56,2	2,4
291,08356	3,166	354944	280588	414277	1016298	995253	1240091	3,10	0,981	3,90E-003	19,1	12,5
165,07524	5,537	414381	423799	468111	1212	440	2329	-328,13	0,002	1,40E-003	6,6	71,6
199,17049	40,49	909796	886744	743696	330822	276070	284220	-2,85	0,072	4,90E-003	10,6	9,9
389,05109	2,211	646802	656037	722490	225731	259486	207655	-2,92	0,06	2,70E-004	6,1	11,4
389,06064	2,285	334287	367450	397524	163504	122330	112866	-2,76	0,105	7,00E-004	8,6	20,3
330,04276	2,339	40933	39225	38030	360784	400981	360139	9,49	0,946	1,60E-003	3,7	6,3
451,30737	30,03	767515	755649	687842	276054	220503	224108	-3,07	0,061	1,60E-004	5,8	12,9
165,07524	4,976	492	13537	0	344804	429982	368446	81,49	143,206	3,60E-003	164,2	11,5
401,1572	14,28	0	3487	0	1253125	1406057	1000976	1049,66	1994,336	9,30E-003	173,2	16,8
476,27905	34,86	803882	801647	754788	224382	217819	180089	-3,79	0,04	1,30E-005	3,5	11,5
225,13498	24,47	1288565	1008903	1181930	336658	252311	495161	-3,21	0,144	1,90E-003	12,2	34,1
254,22104	48,3	677133	637796	795154	216530	198138	180876	-3,54	0,058	6,60E-003	11,6	9
328,23666	47,27	647932	766661	601025	298901	236277	154543	-2,92	0,151	2,60E-003	12,7	31,5
351,01285	2,362	352941	372367	382133	185131	162684	160700	-2,18	0,055	7,10E-005	4	8
141,01714	6,368	564392	707273	742166	190199	244424	292320	-2,77	0,127	5,60E-003	14	21,1
225,11412	24,47	525959	541776	521485	214708	174378	130379	-3,06	0,086	3,00E-003	2	24,4
241,11955	15,32	0	93958	767	4612342	6053566	5908734	174,98	324,531	6,70E-003	171,1	14,4
451,27087	21,8	167277	175163	168818	470656	553578	467156	2,92	0,359	7,10E-003	2,5	9,8
279,24081	49,36	876637	774812	712341	202999	163754	171215	-4,39	0,05	4,10E-003	10,5	11,6
255,08034	13,52	9484	7733	0	351922	306605	317655	56,70	53,976	1,20E-003	87,9	7,3

351,13287	5,742	256210	257044	241696	0	0	0	only CTR	0	3,90E-004	3,4	0
557,45856	48,13	684177	553325	479420	150394	56107	116782	-5,31	0,118	7,20E-003	18,1	44,3
564,33179	35,9	647639	640805	635520	269883	259367	270800	-2,40	0,014	2,10E-007	0,9	2,4
225,12459	25,97	789432	806510	762795	246241	228794	249383	-3,26	0,023	4,50E-005	2,8	4,6
402,16028	14,46	0	12473	0	526781	502659	573234	128,49	231,185	1,10E-003	173,2	6,7
580,39459	40,49	627420	840210	785278	251698	183280	161408	-3,78	0,102	6,00E-003	14,7	23,7
351,13297	5,537	256210	259523	295452	0	0	0	only CTR	0	2,20E-003	8	0
225,12463	26,2	688877	686238	611693	214000	214109	211361	-3,11	0,024	3,10E-003	6,6	0,7
241,11407	15,51	0	6105	6066	461242	447928	520297	117,45	111,208	2,10E-003	86,6	8,1
523,15985	15,12	0	0	703	436257	399471	405497	1765,61	3142,168	7,50E-004	173,2	4,8
588,33167	35,9	605075	598271	575389	263084	263606	272325	-2,23	0,021	2,40E-004	2,6	1,9
554,3476	39,16	665017	705937	658188	317821	289122	248534	-2,37	0,068	1,70E-004	3,8	12,2
141,01715	6,819	670455	831003	614407	216173	268674	363159	-2,50	0,169	8,30E-003	15,9	26,4
567,3186	19,37	5163	3755	2982	347086	312125	353929	85,14	29,381	1,50E-003	27,9	6,6
213,97603	13,44	1874	337	855	322291	277785	246736	276,19	248,55	6,00E-003	76,5	13,5
605,15656	2,286	195495	181193	142541	41724	8040	72940	-4,23	0,225	6,20E-003	15,8	79,4
565,30249	17,51	5860	3738	6544	285168	276034	333566	55,43	20,819	3,60E-003	27,2	10,4
480,31058	41,7	564163	654661	717304	202551	156653	176182	-3,62	0,069	5,50E-003	11,9	12,9
496,27481	20	86103	83137	90588	421959	413873	366571	4,63	0,546	2,70E-003	4,3	7,5
1105,7422	42,19	622789	569268	524786	298074	215437	251049	-2,25	0,111	1,20E-003	8,6	16,3
391,28589	30,03	599482	579881	517056	219681	169750	170934	-3,03	0,076	5,00E-004	7,6	15,3
193,01353	2,749	39078	37986	50186	508928	489241	397207	10,97	3,151	6,10E-003	15,9	12,8
288,61972	17,25	11581	7056	8325	258306	264255	279835	29,76	8,967	3,80E-004	26	4,2
143,10806	20,54	88567	67095	84936	478954	507089	411007	5,81	1,448	3,80E-003	14,3	10,6
283,16422	3,636	176374	237681	217860	81104	122130	85906	-2,19	0,174	8,80E-003	14,9	23,3
310,12573	2,749	66309	71590	88582	358461	342911	304102	4,44	1,055	1,20E-003	15,4	8,4
583,38245	41,69	610702	566364	709030	352693	281778	263790	-2,10	0,13	4,80E-003	11,6	15,7
255,08055	14,13	0	7039	0	661104	497039	612574	251,56	471,664	6,70E-003	173,2	14,3
331,03751	2,339	21491	38015	32153	245122	258642	229712	8,00	2,668	1,30E-004	27,4	5,9
554,34747	38,98	416266	537236	445447	242597	196410	170366	-2,30	0,137	6,80E-003	13,5	18
484,25037	15,52	0	728	522	337339	299931	348175	788,36	770,636	2,00E-003	90,1	7,7
684,72498	15,62	0	375	343	308206	335758	278205	1284,36	1236,713	2,90E-003	86,9	9,4
255,58221	14,34	0	390	0	307906	317831	331042	2453,28	4334,889	4,40E-004	173,2	3,6
373,00317	2,356	58809	54749	53116	226998	210229	192565	3,78	0,509	3,30E-003	5,3	8,2
466,28983	21,84	145679	140258	110327	429795	396061	347578	2,96	0,74	2,90E-003	14,4	10,6
223,17067	38,58	531255	439122	481154	283899	220685	177150	-2,13	0,156	3,50E-003	9,5	23,6
248,03462	5,41	193884	153097	213180	326	1329	1077	-205,04	0,004	8,90E-003	16,4	57,3
291,08359	3,345	195496	162085	175736	849848	820216	846975	4,72	0,538	1,10E-006	9,4	1,9
225,12453	27	528494	590887	572718	183614	180554	185918	-3,08	0,023	2,20E-003	5,7	1,5
178,04721	14,78	4117	0	472	264399	271154	284624	178,73	270,072	2,80E-004	147,3	3,8
310,0567	2,211	403422	400588	454770	131852	109636	99543	-3,69	0,059	5,30E-004	7,3	14,5
341,60776	14,53	0	270	0	214712	193289	232601	2372,60	4336,277	2,80E-003	173,2	9,2
313,07364	2,3	73372	62863	69124	206486	235313	213150	3,19	0,467	1,40E-003	7,7	6,9
186,02647	13,86	247	0	485	269281	250999	306896	1130,02	1241,625	3,50E-003	99,4	10,3

437,29153	30,03	435045	434229	387433	168655	133172	139787	-2,85	0,068	2,90E-004	6,5	12,8
211,07253	4,699	43891	2519	0	254588	215755	238045	15,26	25,577	3,50E-004	159,3	8,3
405,22867	25,11	37447	36328	43478	421037	395001	331563	9,79	2,141	5,70E-003	9,8	12
498,29004	22,06	62052	48667	46276	288059	262545	223019	4,93	1,427	5,70E-003	16,2	12,7
241,21758	49,9	482943	546087	624424	108099	87726	55605	-6,58	0,068	3,50E-003	12,9	31,6
225,12456	27,86	435583	469519	407541	119680	129044	126815	-3,50	0,031	2,70E-003	7,1	3,9
340,20352	31,74	397077	401032	404538	110782	114143	78742	-3,96	0,051	1,00E-003	0,9	19,3
151,06136	3,26	93756	87635	73956	550140	494539	482854	5,98	1,135	1,30E-003	11,9	7,1
329,0307	1,879	384606	321210	376997	143982	171137	154350	-2,31	0,08	4,30E-003	9,6	8,8
297,24368	37,97	467857	386355	405878	120754	111505	77068	-4,07	0,08	1,30E-003	10,1	22,3
555,44299	46,29	345340	301549	311592	5586	1841	2968	-92,21	0,007	1,60E-003	7,2	55,4
145,03825	3,26	178571	142285	119985	386245	389506	353682	2,56	0,65	7,40E-004	20,1	5,3
141,01717	8,078	428654	526789	416880	207481	138783	208364	-2,47	0,141	4,60E-003	13,2	21,6
515,28888	18,21	89516	76772	91889	358280	356946	321332	4,01	0,623	6,60E-004	9,4	6,1
335,04959	2,855	55530	65925	73247	210480	208912	226619	3,32	0,606	4,20E-005	13,7	4,6
205,97871	12,11	353	525	697	401178	388664	325380	708,08	309,617	4,00E-003	32,8	10,9
172,99156	5,483	0	184970	0	1377433	1212375	1787408	23,66	45,79	8,30E-003	173,2	20,3
130,05933	4,535	44287	50832	73331	166074	213385	172685	3,28	1,346	3,80E-003	27,1	13,9
171,13924	32,4	464011	389833	510256	229188	181715	180670	-2,31	0,119	8,50E-003	13,4	14,1
1101,7106	38,08	378256	293884	356564	188876	132448	120263	-2,33	0,162	4,50E-003	12,8	24,9
311,04984	2,356	74184	82440	66650	214202	211839	204590	2,82	0,367	5,70E-005	10,6	2,4
246,07503	5,596	189142	232437	195842	1050	461	876	-258,66	0,002	4,30E-003	11,3	38,1
499,29364	19,92	27682	33289	33708	236884	230358	200648	7,05	1,364	2,70E-003	10,7	8,7
189,04073	2,712	8300	6061	5991	148354	174629	175240	24,48	7,002	2,90E-003	19,4	9,2
583,31299	17,94	10268	12122	6906	180327	210946	180509	19,52	7,09	2,60E-003	27,1	9,2
576,33142	33,37	332783	403798	299578	67624	147979	140078	-2,91	0,181	5,30E-003	15,4	37,4
141,0172	6,577	727401	707273	665741	150397	266162	275220	-3,04	0,114	2,40E-003	4,5	30,2
299,22336	48,51	342208	422083	363015	134251	126964	121635	-2,94	0,054	8,00E-003	11	5
516,28094	20	24919	21584	22640	190460	217812	189475	8,65	1,337	2,50E-003	7,4	8,1
228,51152	13,2	0	386	237	215081	197532	182714	955,58	974,26	2,20E-003	93,8	8,2
326,18793	30,43	450614	342119	404939	109610	192664	176573	-2,50	0,165	4,70E-003	13,6	27,6
291,0835	3,793	77119	84510	68830	546304	498900	446886	6,47	1,308	3,90E-003	10,2	10
491,2298	18,91	11391	13579	12825	259389	244376	244246	19,79	2,437	3,70E-004	8,8	3,5
227,12912	24,47	438112	402142	344253	118758	134862	115425	-3,21	0,064	7,70E-003	12	8,4
476,27878	34,67	591166	512439	452901	147674	72531	105211	-4,78	0,101	2,60E-003	13,4	34,7
319,22815	37,32	2895	9179	10640	305405	275847	228451	35,65	24,504	6,70E-003	54,4	14,4
239,5648	17,02	176501	164964	166580	0	0	802	-633,47	0,003	4,30E-004	3,7	173,2
321,08063	2,764	277390	243249	290151	70424	59620	81959	-3,82	0,065	1,40E-003	9	15,8
348,10269	15,4	0	1332	0	264372	313841	267290	634,76	1162,263	3,20E-003	173,2	9,8
1106,7455	42,19	360964	319525	298859	177045	119094	141242	-2,24	0,133	2,00E-003	9,7	20,1
199,03807	2,804	289315	272851	274131	155655	146687	105179	-2,05	0,113	6,60E-003	3,3	19,8
324,22641	46,29	310583	294465	286008	33490	21879	26293	-10,91	0,024	8,60E-005	4,2	21,5
225,1246	28,9	413360	539816	510186	137064	143029	176806	-3,20	0,086	7,70E-003	13,6	14,1
225,18633	42,28	351296	344820	311621	84709	66718	82366	-4,31	0,044	4,70E-004	6,3	12,6

151,06145	3,496	65988	67278	93510	345434	355336	415404	4,92	1,512	1,90E-003	20,5	10,2
141,01715	14,42	327199	293862	282088	124575	91419	103572	-2,83	0,083	5,20E-004	7,8	15,7
1021,3087	2,23	67229	52096	35685	168376	154430	130799	2,93	1,261	2,50E-003	30,5	12,6
562,31573	33,37	296529	380496	283512	62494	140163	129732	-2,89	0,189	6,60E-003	16,4	38
607,42242	44,49	338072	333586	262931	132797	103296	95718	-2,82	0,111	6,00E-003	13,5	17,7
505,229	15,52	0	3895	3114	243434	181324	200499	89,21	92,301	7,60E-003	88,2	15,3
186,05629	15,7	870	644	2113	228035	287595	241415	208,72	162,418	5,10E-003	65,4	12,4
301,10428	5,715	121181	161838	159138	0	3703	1950	-78,22	0,015	7,70E-003	15,4	98,3
352,02463	2,356	37556	37429	39292	172781	179210	179141	4,65	0,224	8,60E-005	2,7	2,1
171,03941	13,89	11082	2692	5022	227716	290149	290309	43,00	35,482	5,70E-003	69,1	13,4
621,1214	2,211	276204	281217	275995	79435	57799	65911	-4,10	0,042	4,30E-004	1,1	16,1
474,24167	24,47	329859	281959	276974	127542	97685	94680	-2,78	0,097	1,50E-003	9,9	17
151,04036	3,8	40745	46853	58677	332885	279583	337756	6,50	1,876	2,80E-003	18,7	10,2
143,10797	23,42	82980	98842	91973	266673	342492	298055	3,31	0,706	8,50E-003	8,7	12,6
256,10214	6,152	116124	1835	98479	360598	252960	347734	4,44	4,602	7,30E-003	85,3	18,3
214,02776	4,623	9567	4111	3023	179234	200695	222333	36,06	26,586	3,30E-003	63	10,7
241,11411	14,66	0	9334	0	211320	247870	180869	68,57	129,548	7,20E-003	173,2	15,7
366,99509	2,356	18456	12036	13333	167426	171453	145422	11,05	3,527	1,90E-003	23,2	8,7
267,07245	2,661	63617	54721	54829	182917	182470	168350	3,08	0,416	1,10E-004	8,8	4,7
173,99486	5,716	5911	49201	18086	368012	309299	348674	14,02	14,051	2,00E-004	91,5	8,7
291,0835	9,536	34420	40746	43383	281996	333145	358700	8,21	1,946	5,70E-003	11,7	12
330,15427	5,537	133938	118798	99081	0	0	0	only CTR	0	7,30E-003	14,9	0
516,29095	19,97	13391	10460	17932	163493	160207	179560	12,04	3,999	4,80E-004	27	6,2
291,08368	11,28	24751	18784	49856	325198	435132	422328	12,66	8,639	5,90E-003	53	15,2
141,01717	14,23	288818	312228	300967	53587	91382	67381	-4,25	0,073	2,10E-004	3,9	27
604,36255	36,83	260366	261180	269620	121757	95456	92363	-2,56	0,069	1,60E-003	1,9	15,7
391,28589	30,21	470926	374790	459581	244327	149885	155280	-2,38	0,173	4,30E-003	12,1	29
141,01717	13,93	422154	337524	417541	183197	164783	236106	-2,02	0,155	5,70E-003	12,1	19
291,08347	10,36	39085	32198	56980	300889	337859	362937	7,81	3,067	1,20E-003	29,9	9,3
478,29483	37,68	306432	386658	377997	159837	129131	123241	-2,60	0,103	5,60E-003	12,3	14,3
567,31824	17,9	0	557	0	180958	172336	170140	939,74	1658,724	3,50E-004	173,2	3,3
606,37823	39,35	380551	301808	299414	170729	100931	82141	-2,77	0,193	5,30E-003	14,1	39,6
250,1451	42,42	268036	268888	280292	87118	85458	65025	-3,44	0,052	1,20E-004	2,5	15,5
225,12465	30,72	327692	333837	324827	112022	121797	100226	-2,95	0,038	1,40E-004	1,4	9,7
466,30869	40,4	436586	349856	374382	81673	70536	49917	-5,74	0,062	3,00E-003	11,6	23,9
377,07062	17,77	13848	7285	14399	260078	223734	199751	19,24	8,993	5,90E-003	33,4	13,3
339,2002	32,48	444116	461045	499464	107235	174525	193018	-2,96	0,117	1,30E-003	6,1	28,5
270,09137	2,663	36690	39262	35794	173186	147827	133582	4,07	0,735	9,70E-003	4,8	13,2
298,15649	26,78	252371	317522	274055	57820	58098	66254	-4,63	0,042	6,60E-003	11,8	7,9
399,2757	45,92	54054	51006	31376	215785	211475	211397	4,68	1,322	1,20E-003	27,1	1,2
353,01956	2,339	24100	22560	27124	123406	131922	140923	5,37	0,863	1,20E-003	9,4	6,6
202,02618	15,36	0	16404	0	170327	176073	207278	33,75	62,099	9,90E-004	173,2	10,8
522,65802	14,88	0	348	0	340006	340343	335135	2918,06	5073,978	2,30E-005	173,2	0,9
291,08377	11,6	23841	52506	49495	261603	290742	366256	7,30	4,029	9,20E-003	37,6	17,6

897,24799	2,285	76369	85066	92507	0	0	7256	-35,00	0,052	5,60E-004	9,5	173,2
200,04843	5,742	114721	108907	107947	0	0	0	only CTR	0	3,70E-004	3,3	0
225,12459	32,39	381423	299724	366713	98722	85798	84944	-3,89	0,054	7,70E-003	12,5	8,6
225,12459	31,81	425193	394009	323345	108716	108509	88137	-3,74	0,068	8,80E-003	13,7	11,6
826,5611	43,7	44014	55168	91745	329157	278429	232785	4,40	2,484	6,20E-003	39,2	17,2
594,71527	15,68	0	399	0	129922	93751	116204	851,82	1614,215	8,60E-003	173,2	16,1
310,05698	2,018	252517	257891	287330	132807	134640	121015	-2,05	0,062	2,50E-003	7	5,7
564,3313	36,1	287508	283916	297105	81954	127428	127941	-2,57	0,1	4,90E-003	2,4	23,5
254,22096	48,51	250317	297632	269395	96850	88869	77602	-3,10	0,064	2,00E-003	8,7	11
179,05638	2,575	275107	459173	247355	900584	856790	681277	2,48	1,229	6,80E-003	35,2	14,3
871,53497	46,21	271733	253103	213706	158510	110798	91067	-2,05	0,2	9,30E-003	12	28,9
324,05643	2,339	25035	39024	42416	93477	106146	123891	3,04	1,219	4,40E-003	26	14,2
220,9682	16,59	1035	596	688	213048	210076	180953	260,49	100,876	2,60E-003	29,9	8,8
166,03903	2,737	39831	26140	36028	206295	198170	150548	5,44	2,017	9,90E-003	20,8	16,3
451,30725	30,21	459934	372659	447051	204424	140726	149408	-2,59	0,124	2,10E-003	11	21
146,04599	2,356	7011	11518	0	104321	100406	71590	14,91	16,908	8,80E-003	94	19,4
320,1076	3,644	6765	9459	11704	144722	161118	158910	16,64	5,376	6,00E-004	26,6	5,7
246,07498	5,911	178049	146580	134189	0	0	0	only CTR	0	7,20E-003	14,8	0
354,02515	2,356	24550	16898	18340	85949	83199	76128	4,10	1,091	1,10E-004	20,4	6,2
621,12134	2,008	260457	233448	248462	104056	96508	99167	-2,48	0,038	1,50E-003	5,5	3,8
337,10031	2,749	14038	13448	19213	160272	142092	133676	9,34	2,776	2,40E-003	20,4	9,4
276,12769	18,89	2079	3500	2998	128719	142332	126270	46,32	14,701	1,40E-003	25,2	6,5
1046,6027	19,97	2692	1122	1786	113061	109081	122450	61,53	29,667	1,10E-003	42,2	6
609,4895	49,28	179752	178106	151608	29859	11447	12341	-9,50	0,071	3,70E-004	9,3	58,1
826,56097	42,96	45970	50032	86478	282571	258520	202913	4,08	2,167	5,50E-003	36,7	16,5
826,56097	45,65	44054	70136	104033	300383	266402	217816	3,60	2,057	4,20E-003	41,3	15,9
301,10434	5,933	121181	149371	163263	686	433	429	-280,24	0,002	7,30E-003	14,8	28,5
283,15533	19,37	2139	2244	2274	136432	108639	147937	59,04	10,996	8,10E-003	3,2	15,4
406,26825	21,89	61339	60035	62875	219890	235331	184865	3,47	0,501	9,40E-003	2,3	12,1
321,08063	3,046	280399	266185	236760	116836	119500	95711	-2,36	0,086	1,50E-003	8,5	11,8
329,06033	2,3	49351	36706	30018	135003	122371	123335	3,28	1,014	4,00E-004	25,4	5,5
292,08701	2,816	67026	75217	63264	239315	249120	203564	3,37	0,65	4,90E-003	8,9	10,4
214,0722	5,742	103502	101898	79163	9368	9370	10379	-9,77	0,021	8,30E-003	14,4	6
200,04852	5,537	114721	110759	121126	0	0	0	only CTR	0	6,80E-004	4,5	0
347,08478	2,827	16913	25220	25830	179278	152196	161987	7,26	2,202	1,20E-003	22	8,3
581,39752	40,65	235659	278160	204239	117271	89018	77638	-2,53	0,147	8,70E-003	15,5	21,6
291,08353	7,364	39744	12642	19644	388008	362720	284332	14,37	10,671	6,40E-003	58,6	15,7
826,56104	46,78	34217	64512	108861	277357	256451	207892	3,57	2,453	4,00E-003	54,3	14,4
826,56122	43,16	44734	52322	88087	295159	262085	204776	4,12	2,285	7,70E-003	37,5	18
826,56104	44,04	38695	54295	88029	202557	277846	228174	3,91	2,27	4,40E-003	41,8	16,2
155,04655	2,816	14193	6922	7661	96724	109335	102559	10,72	5,132	9,50E-005	41,7	6,1
225,12466	33,86	263276	261878	268892	77861	77991	76624	-3,42	0,007	7,30E-005	1,4	1
514,26538	18,56	24153	19351	20602	149841	124846	131653	6,34	1,344	3,30E-003	11,7	9,5
188,98643	5,596	103705	82484	98418	47108	46789	34985	-2,21	0,126	4,30E-003	11,6	16,1

1167,856	2,219	19731	32428	33212	133917	118336	115883	4,31	1,49	2,70E-004	26,6	8
826,56122	46,01	29410	61912	102286	288658	258053	209208	3,90	2,83	4,00E-003	56,6	15,9
417,11975	13,97	0	12115	0	150120	147191	135392	35,72	63,794	2,30E-005	173,2	5,4
291,0835	8,342	13873	11711	21055	281093	393412	350368	21,97	10,559	9,50E-003	31,5	16,6
476,27905	35,05	201935	231534	258585	102370	53902	78623	-2,95	0,147	2,30E-003	12,3	31
221,03047	2,892	29962	32348	30353	151072	146026	116186	4,46	0,795	1,00E-002	4,1	13,7
225,12459	34,28	239231	252976	261928	75774	74886	82920	-3,23	0,032	4,00E-004	4,5	5,7
826,56116	46,43	37848	74676	90239	287375	251163	203339	3,66	2,08	5,70E-003	39,8	17
225,12466	34,75	213247	238488	216562	71410	69127	60983	-3,32	0,043	7,80E-004	6,2	8,2
594,3786	42,71	263584	227365	239203	42632	21480	23578	-8,33	0,057	2,20E-004	7,6	39,9
514,27551	18,54	12894	15017	17024	114221	121427	118158	7,87	1,327	1,70E-005	13,8	3,1
881,51868	44,49	224039	228372	199063	65859	33868	72982	-3,77	0,115	6,50E-004	7,3	36,2
199,01105	2,892	4660	1581	3409	158465	159209	118847	45,24	28,952	8,40E-003	48,1	15,9
256,07837	14,34	0	317	0	146008	143733	118653	1288,31	2373,908	4,10E-003	173,2	11,2
616,36285	38,66	235177	226854	222719	77975	63372	51805	-3,55	0,065	3,70E-004	2,8	20,4
200,04854	4,976	0	1852	0	85476	108910	90414	153,78	286,447	5,40E-003	173,2	13
315,06931	2,339	54471	49954	52326	149708	146724	147753	2,83	0,152	2,00E-006	4,3	1
174,98714	5,669	0	24343	0	199380	242342	191617	26,02	48,431	1,50E-003	173,2	12,9
157,01216	2,629	57277	79934	68848	195848	214261	211217	3,02	0,641	1,00E-004	16,5	4,8
226,12799	24,99	435866	326378	363792	146445	104771	105086	-3,16	0,111	7,30E-003	14,8	20,2
397,01849	2,429	202863	210560	200514	87485	97056	90955	-2,23	0,035	1,10E-005	2,6	5,3
307,06482	2,715	152649	149404	147781	45193	62366	44395	-2,96	0,073	2,20E-003	1,7	20
327,02365	2,356	6940	8570	7155	92495	106182	85066	12,52	2,885	4,80E-003	11,7	11,3
484,25021	14,66	0	1337	0	128896	129387	104429	271,29	501,921	4,50E-003	173,2	11,8
481,09314	2,3	10172	20016	16916	61692	71563	54339	3,98	1,827	3,00E-003	32,1	13,8
253,21761	49,11	255454	195684	189797	65600	44578	33268	-4,47	0,115	7,00E-003	17	34,3
239,16544	26,82	233210	201179	232770	24210	21877	20428	-10,03	0,017	2,60E-003	8,3	8,6
282,14731	17,51	1692	1039	1178	105561	110906	142644	91,87	39,621	9,40E-003	26,4	16,7
225,12471	34,46	222672	245451	247973	67440	66227	73174	-3,46	0,032	1,20E-003	5,8	5,4
452,31046	30,03	211628	210006	184983	68984	57725	61599	-3,22	0,051	1,40E-003	7,4	9,1
588,3313	36,1	242169	238241	255636	71650	114734	119030	-2,41	0,122	6,00E-003	3,7	25,7
288,10635	2,749	1702	2605	3159	152999	150668	140557	59,50	20,241	6,00E-004	29,6	4,5
826,56104	46,96	34217	76799	108360	284576	239697	201984	3,31	2,25	6,40E-003	50,9	17,1
240,10722	16,86	164944	166833	164984	9398	8591	4876	-21,73	0,015	4,70E-006	0,7	31,6
312,22626	32,25	202160	202462	242519	40825	25545	22431	-7,29	0,06	1,70E-003	10,8	33,3
291,08344	12,61	18993	28868	32858	203120	259100	242738	8,73	3,387	4,30E-003	26,5	12,2
913,21112	2,211	169578	163857	190493	60957	51485	41062	-3,41	0,081	4,30E-004	8	19,4
158,08241	3,881	588	2327	6883	129088	152744	152768	44,36	48,336	2,00E-003	99,5	9,4
291,08353	6,471	12901	17934	25390	246244	219480	225827	12,30	4,87	2,70E-004	33,5	6,1
826,56134	45,83	42619	71322	104723	188261	261762	217532	3,05	1,81	6,30E-003	42,6	16,6
302,22104	45,33	192954	183322	178704	57271	42387	42246	-3,91	0,057	3,70E-005	3,9	18,3
223,01999	4,349	235016	281873	274001	91450	61551	66736	-3,60	0,087	8,80E-004	9,5	21,8
368,10907	3,582	9212	9833	22692	105902	121119	106646	7,99	4,99	1,40E-004	54,7	7,7
226,05084	14,14	3195	3205	3466	84204	96006	73632	25,73	4,604	6,20E-003	4,7	13,2

249,18626	41,77	194326	230955	176396	24724	19885	13692	-10,32	0,041	6,10E-003	13,9	28,5
826,56116	44,29	32251	42150	71821	252872	250281	203130	4,83	2,615	1,10E-003	42,2	11,9
214,07225	5,537	108215	107253	93603	13770	7378	9344	-10,14	0,04	7,70E-004	7,9	32,2
225,12474	35,61	180546	233468	216685	59822	80926	59115	-3,16	0,1	4,60E-003	12,9	18,6
826,5611	41,22	33545	34024	54950	238534	240119	194325	5,49	2,28	2,00E-003	29,9	11,6
207,07758	6,105	98624	102116	116103	674	330	0	-315,58	0,003	2,50E-003	8,8	100,7
876,2677	2,219	48209	52640	43806	116304	113959	93039	2,23	0,47	9,00E-003	9,2	11,9
225,1246	34,09	256176	262768	268476	77748	85667	74468	-3,31	0,029	3,10E-006	2,3	7,3
223,02017	5,207	286484	214509	284426	26181	21942	17684	-11,94	0,029	9,00E-003	15,7	19,4
351,10129	11,81	0	613	0	177579	162071	158125	812,03	1456,593	1,30E-003	173,2	6,2
630,37848	38,66	212724	210318	203849	72696	56601	44112	-3,62	0,075	1,40E-003	2,2	24,8
388,97745	2,339	1793	7134	0	89595	81178	92564	29,50	38,781	1,10E-004	124,7	6,7
386,99817	2,415	54139	49645	58223	134097	142711	136681	2,55	0,285	1,90E-005	7,9	3,2
225,12473	35,85	221194	216899	227801	66711	66071	64472	-3,38	0,013	2,40E-004	2,5	1,8
223,02023	5,41	248875	208912	229238	27215	33887	37763	-6,95	0,036	2,00E-003	8,7	16,2
350,11765	3,507	3011	0	1549	73708	84838	60104	47,95	55,648	9,10E-003	99,1	17
697,21246	16,41	0	1379	0	223689	255000	258393	534,50	967,738	2,00E-003	173,2	7,8
1102,7139	38,08	203447	170175	204747	109437	83817	74981	-2,16	0,14	2,60E-003	10,2	20
586,31549	33,21	187849	183857	173166	27690	43505	50552	-4,48	0,074	1,80E-004	4,2	28,8
320,99741	3,434	256492	192325	210340	65103	99858	74536	-2,75	0,137	7,00E-003	15,1	22,5
241,13124	15,52	0	3761	2260	167538	148292	184022	83,02	87,215	3,60E-003	94,3	10,7
237,02278	17,7	105193	108796	79869	13218	12978	2054	-10,40	0,081	4,70E-003	16,1	67,7
600,33142	33,23	192207	190701	173084	26134	46994	50013	-4,52	0,083	1,50E-004	5,7	31,7
367,10687	5,537	75355	80909	95373	0	0	0	only CTR	0	5,00E-003	12,3	0
367,10672	5,742	75355	80582	76548	0	0	0	only CTR	0	4,20E-004	3,5	0
348,00879	11,77	1308	1388	664	140313	190093	155500	144,61	74,083	8,30E-003	35,5	15,8
567,31805	18,89	2483	2475	915	108553	104573	91276	51,83	28,518	2,50E-003	46,1	8,9
614,3468	37,62	234002	182047	173257	16571	27439	28366	-8,14	0,054	9,70E-003	16,7	27,2
250,145	42,61	28011	23300	40102	196332	182576	184484	6,16	1,998	2,20E-005	28,4	4
300,12051	5,128	79321	60900	76888	0	0	0	only CTR	0	6,30E-003	13,8	0
313,05304	2,827	66802	61425	67391	204307	193779	164243	2,87	0,463	8,20E-003	5	11,1
207,07764	5,902	98624	99572	106259	647	360	424	-212,76	0,002	5,60E-004	4,1	31,6
687,07623	2,339	41138	58943	34046	155725	169233	134927	3,43	1,37	1,30E-003	28,7	11,3
223,0202	5,019	221127	224119	229109	25237	10626	18390	-12,43	0,034	2,00E-005	1,8	40,4
826,56134	41,65	23257	31690	41690	213061	207462	170686	6,12	2,467	2,50E-003	28,6	11,7
225,12465	36,1	219162	235073	223865	66421	58352	68870	-3,50	0,035	2,90E-005	3,6	8,5
389,05127	2,018	294212	278244	295856	128214	174281	127335	-2,02	0,109	5,80E-003	3,4	18,7
225,12474	34,93	252358	226708	200401	63497	79938	81103	-3,03	0,081	4,60E-003	11,5	13,2
291,08359	6,23	19468	25026	35480	223476	197928	241826	8,29	3,356	1,70E-003	30,5	10
449,2916	24,72	33208	26272	30120	130910	131351	138968	4,48	0,673	1,10E-005	11,6	3,4
247,08276	16,86	95901	98030	86823	6932	4058	4382	-18,26	0,02	8,00E-004	6,4	30,7
313,0817	2,3	33664	20291	28692	72286	92564	79478	2,96	1,099	2,80E-003	24,5	12,6
225,12469	39,31	161937	204388	197350	52860	51971	51365	-3,61	0,038	9,20E-003	12,1	1,4
225,12476	35,12	232774	209114	194186	59827	74958	63938	-3,20	0,066	2,30E-003	9,2	11,8

399,27588	46,52	214018	210416	161018	80404	73366	44359	-2,95	0,149	5,30E-003	15,2	28,9
373,23886	47,27	171035	194804	156129	76101	58820	37030	-3,04	0,149	1,90E-003	11,2	34,2
241,17407	15,51	0	2223	2110	134874	148923	155738	101,44	95,309	1,60E-003	86,7	7,3
329,04749	2,356	5421	4960	5124	93171	84254	71533	16,06	2,831	6,40E-003	4,5	13,1
391,21323	25,06	14208	14581	12816	131497	133412	125239	9,38	0,937	2,60E-004	6,7	3,3
826,56128	47,18	21301	34719	64733	217745	214359	162714	4,93	3,488	2,80E-003	55,2	15,6
291,08353	5,978	23511	21729	35091	259695	199953	260870	8,97	3,731	6,80E-003	27,1	14,5
268,08072	2,022	72372	96130	94242	197185	192229	190236	2,21	0,373	3,20E-003	15,1	1,9
568,36285	43,93	205902	207886	189515	109261	85352	89446	-2,12	0,087	4,60E-004	5	13,5
594,37854	42,91	162595	194187	217713	106197	90118	53007	-2,30	0,205	8,40E-003	14,4	32,8
358,10587	11,23	0	14198	0	127613	130067	110711	25,95	47,168	1,60E-004	173,2	8,6
171,13921	30,57	188504	232831	194995	82106	72799	77330	-2,65	0,067	9,40E-003	11,7	6
442,16037	5,759	58449	66244	70958	0	0	0	only CTR	0	3,10E-003	9,7	0
223,02008	4,642	221428	224851	221588	21503	8920	10687	-16,25	0,031	1,40E-004	0,9	49,7
514,28485	17,59	6738	8326	5947	96168	89492	96851	13,45	2,906	2,90E-004	17,3	4,3
568,32123	19,4	879	490	572	103508	107674	94661	157,57	60,289	1,40E-003	31,7	6,5
214,0723	4,976	15074	13464	13431	113479	93665	98567	7,28	1,227	4,30E-003	6,7	10,1
183,01613	2,892	5305	2271	5107	128423	141199	118104	30,57	15,013	2,40E-003	40,2	9
325,2478	49,45	113272	146189	115761	59854	42660	33998	-2,75	0,159	5,00E-003	14,7	28,9
709,05847	2,356	45080	35752	31564	108819	112924	99808	2,86	0,707	2,30E-004	18,5	6,3
549,30725	19,54	0	672	0	94380	94114	100962	430,74	763,584	4,80E-004	173,2	4
1167,3546	2,021	104445	87104	91372	202518	209010	198775	2,16	0,262	2,50E-004	9,6	2,5
241,06555	15,52	0	2131	2024	152131	148133	158900	110,51	99,739	2,40E-004	86,7	3,6
510,25381	19,04	22560	20519	21933	99481	89801	98591	4,43	0,461	1,20E-003	4,8	5,6
225,12471	38,75	189062	201320	198455	61442	55025	53187	-3,47	0,031	2,00E-005	3,3	7,7
349,00281	17,09	3955	3741	2906	95912	88172	87577	25,62	5,336	8,20E-004	15,7	5,1
145,03828	3,492	76874	60383	74017	165305	203383	218061	2,78	0,734	9,70E-003	12,5	13,9
195,01051	2,737	11724	14732	7274	140856	147229	110874	11,83	5,674	6,70E-003	33,4	14,6
288,61975	16,86	9033	4959	6334	107049	97308	89092	14,44	5,743	2,20E-003	30,6	9,2
225,12473	36,62	188932	164988	195855	48749	52517	54884	-3,52	0,042	3,90E-003	8,8	5,9
697,21222	18,08	0	1234	0	171946	169679	204335	442,43	813,666	3,70E-003	173,2	10,7
225,12459	36,41	180236	211368	180533	59665	56850	52976	-3,38	0,045	4,70E-003	9,4	5,9
218,87421	2,604	130393	145061	151877	19292	23723	17204	-7,10	0,034	1,30E-003	7,7	16,6
589,33484	35,82	253858	227229	228518	111118	110191	113789	-2,12	0,038	4,30E-003	6,3	1,7
329,03961	2,546	234924	78720	270699	712116	670451	789863	3,72	2,26	3,40E-003	52,4	8,4
555,35065	39,09	179503	179575	146344	99816	56738	51597	-2,43	0,204	8,10E-003	11,4	38,2
246,07503	4,976	0	20535	0	122173	121607	108157	17,14	30,844	4,00E-004	173,2	6,8
329,17599	21,89	19425	11063	12591	154740	129965	121842	9,44	4,12	4,40E-003	31	12,6
130,08762	3,166	6710	28358	17148	61540	59000	82605	3,89	3,165	7,30E-003	62,2	19,1
450,96548	2,429	86213	81963	96630	32126	30667	28306	-2,91	0,051	3,70E-003	8,5	6,3
608,3941	42,71	191580	170194	168921	28866	16811	16843	-8,49	0,048	2,80E-004	7,2	33,4
188,98637	5,359	90510	81173	98418	0	9972	7569	-15,40	0,064	4,40E-004	9,6	89
225,12459	39,9	158954	174239	201098	56495	46906	51944	-3,44	0,062	7,10E-003	12	9,3
274,21072	47,27	179531	190059	140441	29145	26120	20921	-6,69	0,047	9,30E-003	15,4	16,4

442,16006	5,537	58449	66244	68531	0	0	0	only CTR	0	2,20E-003	8,2	0
291,08353	5,799	24765	25345	34470	247044	201241	267883	8,47	2,844	7,40E-003	19,3	14,3
452,2738	21,8	38712	47040	43998	115198	140968	118319	2,89	0,606	6,10E-003	9,7	11,3
387,02661	3,272	241538	182293	238392	31208	31193	24578	-7,61	0,037	9,20E-003	15,1	13,2
325,22971	49,36	206779	155013	201122	56166	44422	35357	-4,14	0,092	7,00E-003	15,1	23
566,30554	17,51	1856	281	1970	82325	79156	101070	63,93	52,714	6,00E-003	68,9	13,5
225,12457	41,17	162922	176462	187741	50871	47070	50038	-3,56	0,031	2,60E-003	7,1	4,1
826,5614	47,4	17727	28498	60050	199544	182285	152921	5,03	3,79	1,60E-003	62,1	13,2
225,12457	39,04	171577	186444	201305	52122	58855	46140	-3,56	0,057	1,20E-003	8	12,1
697,21204	18,46	0	212	0	152893	175824	153070	2272,58	4128,461	2,20E-003	173,2	8,2
559,45416	49,36	167076	138130	155852	16433	9557	9332	-13,05	0,034	2,00E-003	9,5	34,3
477,2821	34,86	183770	175809	164414	48969	46226	39786	-3,88	0,041	3,10E-004	5,6	10,5
300,22668	48,21	256095	224695	273913	50570	41195	45940	-5,48	0,037	3,80E-003	9,9	10,2
574,26971	18,51	21229	20503	20116	81353	83519	98884	4,26	0,58	6,50E-003	2,7	10,9
315,19672	18,12	3198	888	3864	97875	120695	101859	40,31	28,351	4,00E-003	58,9	11,4
826,56116	46,61	38698	66231	108861	278199	241489	207774	3,40	2,182	4,00E-003	49,6	14,5
151,06148	5,003	46183	35356	45106	155609	168104	183602	4,01	0,898	1,20E-003	14,1	8,3
498,28973	20,41	6842	7163	8440	106478	100072	84068	12,95	3,005	5,30E-003	11,3	11,9
582,37823	43,61	201570	198914	177073	113513	86659	84026	-2,03	0,119	1,50E-003	7	17,2
322,03976	4,623	0	0	275	59134	59369	62840	659,43	1162,958	3,70E-004	173,2	3,4
582,37842	43,92	191086	180733	160503	94650	80355	68347	-2,19	0,114	1,40E-003	8,8	16,2
405,1243	13,2	0	0	912	108945	98626	106018	343,85	613,153	7,80E-004	173,2	5,1
277,21011	46,29	152529	159695	152716	23350	15740	19134	-7,99	0,028	2,00E-006	2,6	19,6
840,57654	40,24	26433	24827	40500	188030	193334	151389	5,81	2,383	3,70E-003	28,2	12,9
204,06674	6,152	61592	33425	80645	193068	152714	191693	3,06	1,633	3,20E-003	40,6	12,8
835,53534	47,13	148004	146873	111994	64428	60213	25798	-2,70	0,212	7,40E-003	15,1	42,3
288,61963	18,12	2979	3899	5239	71028	87717	63825	18,37	8,203	9,50E-003	28,1	16,5
826,56042	40,86	14375	26239	25130	176168	213978	165662	8,45	3,688	5,40E-003	29,9	13,7
291,0835	13,46	8839	17450	17657	145704	186545	176454	11,58	5,429	4,40E-003	34,4	12,5
225,1246	26,38	642642	655709	613836	198623	251896	179880	-3,03	0,07	3,00E-004	3,4	17,8
225,12477	42,54	133814	161109	152828	55988	44495	56664	-2,85	0,079	2,00E-003	9,4	13,1
365,24686	49,36	178656	152476	174255	65898	97492	76439	-2,11	0,135	2,10E-003	8,3	20,1
530,04022	2,356	3956	0	10733	48068	48792	47091	9,80	11,039	4,40E-003	110,9	1,8
330,03372	2,285	113218	118062	115496	14022	16821	36962	-5,11	0,112	4,70E-003	2,1	55,4
243,06235	3,166	2857	4251	4861	53653	49219	62544	13,82	5,259	5,00E-003	25,7	12,3
697,2121	18,28	0	785	0	154063	169749	174018	634,18	1138,863	1,30E-003	173,2	6,3
359,11975	2,715	81930	85882	75632	43748	39186	37326	-2,02	0,072	7,50E-004	6,4	8,2
452,27896	35,59	131691	150987	120991	72656	50725	42774	-2,43	0,162	3,20E-003	11,3	27,9
840,57635	42,87	27268	32975	51036	175053	159676	134721	4,22	1,96	2,20E-003	33,5	13
313,16461	28,37	175070	153442	193806	43257	52813	38773	-3,87	0,071	4,00E-003	11,6	16
840,57629	43,61	26311	33700	49354	123550	149947	132771	3,71	1,566	7,10E-004	32,3	9,9
164,07188	6,278	190099	154102	137467	5011	1780	2377	-52,54	0,014	9,40E-003	16,8	56,3
362,01221	2,356	7748	6970	6186	44691	46504	38961	6,23	1,263	2,90E-003	11,2	9,1
1047,6062	19,97	1490	647	0	61583	64542	70239	91,89	102,539	1,20E-003	104,9	6,7

830,49829	44,45	17435	20282	31888	150392	148346	143580	6,35	2,248	2,10E-004	33	2,4
389,26981	24,77	14604	5478	10609	102144	129755	96067	10,69	6,533	7,70E-003	44,7	16,4
315,63379	19,08	1220	1290	1032	74891	58283	60581	54,70	13,809	6,60E-003	11,3	13,9
535,38641	44,85	166599	164794	188656	89447	63236	94700	-2,10	0,134	2,30E-003	7,7	20,4
191,0199	2,661	20431	23037	16005	107308	111365	92006	5,22	1,452	2,20E-003	17,9	9,9
168,02458	2,816	4369	0	6687	105199	114381	103554	29,23	28,511	6,90E-005	92,1	5,4
220,87128	2,604	115937	128299	128315	21491	27181	24469	-5,09	0,034	4,50E-004	5,8	11,7
226,12796	25,21	166733	207073	226396	43762	50827	67022	-3,71	0,101	7,30E-003	15,2	22,1
840,57623	43,78	26960	34853	51130	120672	156753	126671	3,58	1,685	3,40E-003	32,7	14,4
358,10495	14,8	702	0	0	79823	69327	72892	316,30	570,843	1,70E-003	173,2	7,2
697,21222	20,86	1045	0	0	147978	166128	153704	447,67	802,101	1,10E-003	173,2	6
225,12474	42,35	134434	150959	176802	42397	42137	55778	-3,29	0,093	7,00E-003	13,9	16,7
497,27798	20	25770	21402	22469	105378	95753	92093	4,21	0,709	1,20E-003	9,8	7
826,56091	44,49	24480	43115	62328	196361	204594	175308	4,44	2,287	5,70E-004	43,7	7,9
255,58189	13,53	1927	781	0	61869	73302	69283	75,50	87,526	2,00E-003	107,4	8,5
890,7439	15,78	0	0	791	109686	140647	116829	464,17	865,043	5,80E-003	173,2	13,2
289,12128	17,25	2635	1491	1570	67108	80116	70386	38,20	16,417	2,80E-003	33,6	9,3
840,5766	37,73	29463	28842	33797	166899	164825	128776	5,00	1,138	9,20E-003	8,8	14
725,03235	2,356	8254	0	7284	85111	84242	63534	14,99	15,414	4,60E-003	87,1	15,7
277,21762	46,96	251273	253022	218430	25525	16032	16684	-12,41	0,029	1,40E-003	8,1	27,3
262,04886	4,976	0	7891	0	100891	92720	82387	34,98	64,104	7,60E-004	173,2	10,1
225,12463	40,78	149320	155691	176885	50295	54420	46574	-3,19	0,053	3,50E-003	9	7,8
265,21762	47,18	130361	132827	125761	18057	13171	9418	-9,57	0,036	5,10E-006	2,8	32
697,21252	19,04	0	319	0	136725	160295	159745	1431,87	2604,747	2,60E-003	173,2	8,8
260,0235	3,166	9493	2733	10320	95839	84432	90070	11,99	7,399	6,80E-005	55,4	6,3
249,02428	2,892	9314	3473	6440	87070	91599	77950	13,35	7,167	6,90E-004	45,6	8,1
270,0701	3,881	778	1574	0	76899	87093	87217	106,81	114,809	1,40E-003	100,4	7,1
697,2121	19,66	0	609	788	146525	159692	149393	326,13	304,218	6,70E-004	88,7	4,6
523,66296	15,07	0	0	222	74902	80200	83684	1075,61	1923,879	1,00E-003	173,2	5,6
413,29138	45,92	25654	24282	12897	104896	96541	104412	4,87	1,851	1,90E-004	33,4	4,6
840,57605	43,06	27508	32975	51974	120268	163555	135444	3,73	1,863	4,80E-003	34,3	15,7
481,31384	41,6	160243	145189	185536	67550	55733	59716	-2,68	0,083	8,50E-003	12,5	9,9
592,36267	39,09	160536	152979	123915	65629	36435	28355	-3,35	0,174	3,00E-003	13,3	45,1
317,21234	31,34	146747	138175	151276	46707	59479	58232	-2,65	0,066	8,70E-005	4,6	12,8
240,95708	15,52	0	2697	1246	116163	108268	125606	88,77	97,76	1,50E-003	102,7	7,4
826,56042	37,51	15713	22676	26942	156620	170648	122394	6,88	2,931	9,40E-003	26	16,6
279,22415	46,29	125113	113229	117369	16216	11762	14333	-8,41	0,025	3,10E-004	5,1	15,9
840,57587	40,93	14869	15072	24290	144368	112228	109104	6,74	3,087	7,90E-003	29,8	16
697,21191	18,71	0	872	434	154821	169136	162441	372,43	389,526	6,30E-004	100,2	4,4
392,28915	30,03	142864	137740	120764	53246	37020	36924	-3,16	0,098	5,50E-004	8,6	22,2
840,57623	43,25	25322	36947	50042	176047	158913	135991	4,19	1,922	2,10E-003	33	12,8
408,9718	2,429	106462	115116	123058	40347	42418	27451	-3,13	0,094	3,10E-004	7,2	22,1
697,21179	21,37	0	969	741	126814	146544	136686	239,79	230,466	1,70E-003	88,9	7,2
225,12466	41,51	208630	170843	166162	44668	48417	44149	-3,98	0,045	9,10E-003	12,8	5,1

465,28632	20,87	35860	52437	46380	107763	92198	88111	2,14	0,631	3,10E-003	18,7	10,8
283,15518	17,9	328	316	0	85541	77268	77741	373,52	345,663	1,10E-003	86,6	5,8
402,16031	14,28	0	763	0	211802	245046	185256	841,55	1575,106	6,50E-003	173,2	14
297,03619	2,71	79214	76674	87198	21834	17840	15465	-4,41	0,055	2,80E-004	6,8	17,5
228,90311	2,601	96821	109820	109470	22038	24910	25277	-4,38	0,033	1,70E-003	7	7,4
188,00232	4,453	900	10741	19847	56335	86447	76756	6,97	7,759	6,80E-003	90,3	21
606,37842	39,09	135923	136316	106363	54745	27273	27078	-3,47	0,165	2,70E-003	13,6	43,8
697,2124	21,55	0	969	741	122463	109404	141252	218,20	222,051	5,50E-003	88,9	12,9
212,9794	15,82	140970	168908	148767	71074	73311	69819	-2,14	0,056	9,50E-003	9,4	2,5
213,14966	23,71	107054	128165	121970	13865	8508	13726	-9,89	0,035	1,90E-003	9,1	25,4
840,57635	42,61	23637	30762	44553	154939	148590	120129	4,28	1,942	2,50E-003	32,2	13,1
287,22308	49,9	131123	150607	179229	30262	24078	16862	-6,47	0,068	7,70E-003	15,7	28,3
165,03075	3,496	365	0	0	38080	46813	53000	377,79	715,248	8,80E-003	173,2	16,3
141,12883	16,63	0	0	310	88374	100493	109817	963,50	1770,712	3,90E-003	173,2	10,8
292,08688	3,005	52886	49152	69747	163313	147914	185830	2,89	0,887	2,70E-003	19,2	11,5
225,12466	42,84	135642	144324	154043	37962	40356	44182	-3,54	0,04	1,10E-003	6,4	7,7
1021,3095	2,021	43234	38489	35198	94860	91820	106978	2,51	0,466	1,60E-003	10,4	8,2
697,21234	17,9	0	914	279	162355	151697	157056	394,89	478,404	3,50E-004	117,8	3,4
331,19147	21	3209	8609	6901	104355	102646	117749	17,35	9,001	8,80E-004	44,2	7,6
626,34692	35,61	124629	150742	147183	47197	71074	42674	-2,63	0,147	2,00E-003	10,1	28,4
181,062	5,902	62031	69049	76188	14389	12509	10806	-5,50	0,045	3,40E-003	10,2	14,3
262,04886	5,596	67102	82168	65552	0	342	0	-628,13	0,003	5,40E-003	12,8	173,2
565,3349	35,95	180967	180945	173452	73699	68429	41953	-2,91	0,104	4,70E-003	2,4	27,7
241,10828	4,203	705	0	0	48840	49770	56386	219,85	398,073	2,00E-003	173,2	8
244,15915	34,26	117146	140002	142366	41482	33029	36208	-3,61	0,061	3,90E-003	10,5	11,6
310,55869	2,149	106129	80317	96210	18983	9026	19865	-5,90	0,087	3,20E-003	13,8	37,7
697,21191	21,17	789	284	297	138257	144443	183429	340,24	268,248	8,20E-003	63,1	15,8
840,57635	36,54	15419	4396	20065	124498	180374	142601	11,22	8,938	9,90E-003	60,5	19,1
608,3941	42,91	112926	141108	143028	77020	59326	38915	-2,27	0,2	7,60E-003	12,7	32,6
405,22885	25,3	14836	20085	28113	183464	153452	138771	7,55	3,486	5,60E-003	31,8	14,4
826,56018	33,83	17179	20233	25034	146686	124647	116410	6,21	1,933	4,70E-003	19	12,1
476,2789	34,35	211360	204505	204886	109251	89739	102577	-2,06	0,057	9,50E-004	1,9	9,9
115,07671	9,693	12101	13208	15436	141968	171087	159342	11,59	2,529	3,10E-003	12,5	9,3
212,97925	15,51	145140	138891	152854	44496	50962	78622	-2,51	0,144	7,30E-003	4,8	31,2
197,04326	3,496	26711	13219	15873	44230	42316	50881	2,46	1,188	8,30E-003	38,4	9,8
303,08353	3,706	29827	34308	36380	73398	66620	75509	2,14	0,353	5,30E-004	10	6,5
271,07068	4,286	0	512	0	110173	118564	125395	691,66	1241,733	1,40E-003	173,2	6,5
697,21271	17,68	521	0	1884	173107	140224	145856	190,93	253,741	4,30E-003	121,4	11,5
199,99678	12,88	2158	1745	3608	61861	61150	56361	23,88	10,525	3,20E-004	39,1	5
840,57611	35,97	19971	22437	28900	156071	160295	129828	6,26	1,908	3,50E-003	19,4	11,1
826,56091	47,61	19845	21265	53216	139209	143807	130824	4,39	2,843	5,60E-003	60	4,8
231,99229	2,892	1127	2371	1189	85435	85805	74933	52,52	27,529	1,70E-003	44,9	7,5
241,28282	15,52	0	1478	1406	103603	96381	110251	107,57	100,463	1,30E-003	86,7	6,7
697,21228	17,07	390	639	0	142516	151488	130875	412,90	417,859	1,80E-003	93,9	7,3

521,20313	15,52	0	1097	605	104663	80179	87907	160,25	177,288	6,30E-003	96,8	13,8
280,2442	49,36	163174	142719	133067	36346	27286	29774	-4,70	0,054	3,30E-003	10,5	15
643,10315	2,293	34088	37867	34441	0	0	0	only CTR	0	1,20E-003	5,9	0
350,10941	3,025	5030	5510	5569	70398	74797	71676	13,46	1,163	3,10E-004	5,5	3,1
697,21301	17,47	0	0	787	121269	143257	145530	521,04	953,6	3,20E-003	173,2	9,8
697,21216	21,73	0	0	370	141436	131761	124974	1076,14	1929,128	1,30E-003	173,2	6,2
580,84283	16,63	0	480	0	104448	135783	127095	765,26	1426,695	5,80E-003	173,2	13,2
419,00012	2,436	96005	107538	109020	35681	36274	33473	-2,96	0,037	2,60E-003	6,8	4,2
215,05382	2,663	15461	17499	10478	63530	58108	46670	3,87	1,561	6,60E-003	24,9	15,3
513,27271	18,25	16102	17977	21244	76757	70258	70377	3,93	0,756	7,50E-005	14,1	5,1
210,07724	5,742	49254	51796	44753	0	0	0	only CTR	0	1,80E-003	7,3	0
657,10724	2,298	0	0	7999	37823	29652	27604	11,89	22,615	2,30E-003	173,2	17,1
295,228	40	130560	127267	120595	12478	8866	9065	-12,44	0,019	1,20E-004	4	20
352,13596	5,742	46916	45756	36390	0	0	0	only CTR	0	5,90E-003	13,4	0
313,1459	2,339	29114	25336	19510	73254	64369	68181	2,78	0,727	3,30E-004	19,6	6,5
318,19431	33,05	106062	91139	99295	50184	55359	39357	-2,05	0,12	1,40E-003	7,6	16,9
312,9852	2,339	30484	18371	19654	74944	69431	68761	3,11	1,055	1,60E-003	29,1	4,8
261,00754	3,041	1843	0	0	83458	80348	83507	134,19	235,359	3,30E-006	173,2	2,2
840,57648	46,01	20825	35163	53243	139348	120510	99940	3,29	2,011	5,30E-003	44,6	16,4
840,57654	44,29	21301	29049	43184	139555	141611	102666	4,10	2,163	6,70E-003	35,6	17,1
567,31793	19,66	4455	4607	4282	78119	72711	67602	16,37	1,781	2,00E-003	3,7	7,2
451,30719	25,62	89077	88442	106294	26389	38131	28537	-3,05	0,101	1,70E-003	10,7	20,2
339,20001	31,16	156573	193951	166463	39260	44651	68356	-3,40	0,123	1,30E-003	11,2	30,5
280,22952	49,36	152821	109800	135327	41080	35994	24069	-3,93	0,107	8,00E-003	16,3	25,9
210,84265	2,601	90697	98368	101219	11958	12005	13632	-7,72	0,017	1,00E-003	5,6	7,6
352,13623	5,537	46916	46101	47783	0	0	0	only CTR	0	1,10E-004	1,8	0
279,2114	49,36	141702	133309	124805	41316	33996	36463	-3,58	0,046	6,20E-004	6,3	10
225,12459	44,8	104282	132149	137246	44747	33903	34315	-3,31	0,092	7,90E-003	14,2	16,3
225,12463	44,07	122634	140105	145125	36077	35154	40227	-3,66	0,044	3,30E-003	8,7	7,3
473,14578	15,77	0	671	0	91134	80096	98995	402,72	739,837	3,70E-003	173,2	10,5
424,95395	2,414	14664	12265	12764	71499	63826	57434	4,86	0,997	5,10E-003	9,6	11
330,03394	2,081	113006	111617	104003	29454	31248	47561	-3,04	0,106	1,70E-003	4,4	27,6
882,52179	44,49	109960	113784	93507	32519	18649	34910	-3,69	0,111	8,00E-004	10,2	30,6
325,19205	30	120174	95081	105430	31333	28345	42399	-3,14	0,107	2,40E-003	11,8	21,8
827,56433	45,65	21013	28562	50257	132529	117863	98084	3,49	2,112	3,60E-003	45,6	14,9
697,21228	17,25	0	1327	258	128331	143205	130090	253,39	352,645	1,10E-003	133,1	6,1
180,06668	5,742	48009	36812	36622	2679	3475	1305	-16,28	0,037	8,30E-003	16,1	44,2
162,95731	2,575	6603	13690	10788	90251	90532	79211	8,37	3,5	3,00E-004	34,4	7,5
498,29034	18,91	4994	6924	6241	65652	66310	61705	10,67	2,136	1,20E-004	16,2	3,9
331,04291	2,356	4622	2325	7895	54508	55283	48365	10,66	6,795	1,10E-004	56,6	7,2
608,42554	44,49	116959	117551	96381	43458	34222	31648	-3,03	0,092	2,60E-003	10,9	17
437,13364	14,46	0	3272	0	92462	88515	105742	87,63	160,06	2,20E-003	173,2	9,4
581,39709	40,83	162871	176031	155075	83140	62952	59346	-2,40	0,105	6,60E-004	6,4	18,7
697,21204	22,28	0	448	0	119937	132315	125834	843,94	1501,839	7,90E-004	173,2	4,9

241,10829	14,66	0	2999	0	88274	88792	71489	82,88	153,415	3,90E-003	173,2	11,9
512,25287	18,54	8950	9617	9047	55422	70275	60153	6,73	1,088	6,70E-003	3,9	12,2
584,3161	17,94	4086	4394	2791	59389	66233	59389	16,41	4,768	1,00E-003	22,6	6,4
1159,7874	40,71	104202	101439	82636	28647	20908	16089	-4,39	0,094	2,10E-003	12,2	29
840,57629	45,74	22234	38798	52084	141250	134532	107370	3,39	1,819	2,90E-003	39,7	14
826,56073	41,84	29580	36023	37512	147811	144104	124946	4,04	0,853	2,10E-003	12,3	8,8
827,56433	43,52	22977	23606	39565	95429	120690	97225	3,64	1,682	2,60E-003	32,7	13,5
582,3783	44,49	122908	136626	109952	57160	47500	49621	-2,39	0,086	5,70E-003	10,8	9,9
840,57611	33,35	14273	16606	18661	129445	119721	99496	7,04	1,861	6,80E-003	13,3	13,1
871,76733	18,69	0	289	0	145547	125187	128847	1382,63	2510,926	2,20E-003	173,2	8,1
397,22668	28,44	66499	83053	85828	11247	8541	9812	-7,95	0,034	6,90E-003	13,3	13,7
840,57599	34,34	23044	23152	26363	138551	129009	107345	5,17	1,064	7,60E-003	7,8	12,8
827,56451	39,96	17864	18780	29817	135142	128672	96952	5,43	2,553	8,80E-003	30	17
210,07732	5,537	49254	53616	51707	2020	1165	3207	-24,18	0,022	7,60E-005	4,2	48,1
222,99187	5,631	834	2369	0	107101	88722	100975	92,66	113,017	2,70E-003	112,5	9,5
281,0882	2,712	26471	17168	12796	59384	67324	56692	3,25	1,5	1,50E-003	37,1	9
697,2121	22,66	1721	0	0	106305	127147	129109	210,67	387,021	3,50E-003	173,2	10,5
345,22864	17,42	3560	309	0	64967	65081	64362	50,25	77,156	2,00E-004	152,9	0,6
225,12465	45,57	105795	121272	130218	34039	31263	30942	-3,71	0,042	5,90E-003	10,4	5,3
241,11937	18,93	92743	80502	75200	24819	29674	28363	-3,00	0,067	5,70E-003	10,9	9,1
452,96252	2,429	60113	53029	57625	19503	19831	18806	-2,94	0,031	2,60E-003	6,3	2,7
225,1246	43,79	130866	140946	147042	40980	40299	42632	-3,38	0,026	1,90E-003	5,9	2,9
200,1738	40,49	118210	113428	91803	39173	26228	31268	-3,35	0,1	4,40E-003	13,1	20,3
438,29462	30,03	107312	105949	93946	40987	31236	31770	-2,95	0,078	3,40E-004	7,2	15,8
283,15512	18,89	1766	0	795	51659	56055	47087	60,45	67,885	1,90E-003	103,6	8,7
225,06178	3,244	69615	52602	46157	151258	122415	157654	2,56	0,888	4,20E-003	21,6	13,1
581,297	18,17	14833	14101	11349	50908	57715	56249	4,09	0,827	4,00E-004	13,7	6,5
840,57617	37,12	23645	28842	27044	137608	162262	126940	5,37	1,218	7,10E-003	10	12,7
291,08334	25,38	2207	5231	8109	99007	120214	87408	19,72	14,444	8,10E-003	57	16,3
593,36615	39,31	94199	104389	99978	45509	34181	18554	-3,04	0,153	7,10E-003	5,1	41,3
151,06145	5,483	44052	46471	37834	126560	140913	161899	3,35	0,764	7,40E-003	10,4	12,4
406,23196	25,16	6725	6168	7286	109716	93988	84838	14,30	3,06	6,40E-003	8,3	13,1
697,21222	23,33	0	0	273	98357	128002	125715	1289,65	2413,214	6,50E-003	173,2	14,1
826,56049	37,96	16729	13656	21207	114524	124717	92217	6,42	2,385	7,90E-003	22,1	15
467,15677	18,73	4338	3217	3363	57842	57682	48405	15,01	4	3,40E-003	16,7	9,9
208,8456	2,601	73924	76735	81683	10284	10715	11911	-7,06	0,018	7,40E-004	5,1	7,7
1107,7482	42,19	115107	91082	98243	56412	42044	49373	-2,06	0,13	6,40E-003	12,2	14,6
610,49304	49,28	69044	67162	62057	13037	4237	5410	-8,74	0,079	1,20E-004	5,5	63,2
840,57635	46,78	19315	34950	51282	121074	116591	89605	3,10	1,893	5,50E-003	45,4	15,6
827,56421	40,15	18883	17020	27151	130176	120718	97803	5,53	2,211	5,80E-003	25,7	14,3
319,14005	15,14	0	2710	0	70412	67311	57538	72,05	132,227	2,40E-003	173,2	10,3
241,13126	14,66	0	4538	0	88446	97524	74043	57,30	107,065	4,70E-003	173,2	13,7
213,14955	23,89	108872	132490	122385	22219	22323	17964	-5,82	0,037	3,40E-003	9,8	11,9
225,12469	45,28	107493	117723	117825	33164	31828	42892	-3,18	0,069	8,90E-005	5,2	16,8

827,56439	42,96	19304	21450	38381	123415	114603	89122	4,13	2,313	4,80E-003	39,6	16,3

Table S2 reports: quantitated ion (m/z), retention time (RT), ion intensity in each replicates, fold change, standard deviation (SD), $p<0.01$ (welch's test), coefficient of variation (CV).

Supplemental Table 3. Features detected in positive and negative ion mode showing significant changes (two-fold increase or decrease in ion intensity, coefficient of variation (CV) <10%, Welch's test p<0.01) after cardiac arrest and cardiopulmonary resuscitation.

Metabolite	Measured Mass	Delta ppm	Adduct	MW	FC CPR2 vs CTR*	Chemical formula	Taxonomy class	HMBD ID	kegg id	metlin ID
1-(beta-D-Ribofuranosyl)-1,4-dihydronicotinamide	255,09882	0,656	-1H	256,255	4,29	C11H16N2O5		HMDB11648		
11beta-Hydroxyprogesterone	331,2269	0,425	+1H	330,2195	7,54	C21H30O3	Not Available	HMDB13594	C05498	
12-KETE	317,21234	0,425	-1H	318,2195	-2,65	C20H30O3		HMDB13633	C14807	
1-Methylinosine	281,0882	3,308	-1H	282,0964	3,25	C11H14N4O5	Nucleoside Analogues	HMDB02721		3780
2-Methyl-3-ketovaleric acid	129,05615	3,386	-1H	130,063	2,88	C6H10O3	Keto-Acids	HMDB00408	c03467	
2-Piperidinone	100,07542	2,750	+1H	99,06841	-5,42	C5H9NO		HMDB11749		
3 -Ketolactose	341,10648	3,985	+1H	340,1006	12,85	C12H20O11	Carbohydrates	HMDB01030	C05403	
3b-Hydroxy-5-cholenoic acid	375,28937	0,033	+1H	374,2821	-5,73	C24H38O3	Bile Acids	HMDB00308		5297
3-Chlorotyrosine	214,02776	0,487	-1H	215,0349	36,06	C9H10ClNO3	Amino Acids	HMDB01885		6369
3-Dehydroquinate	189,04073	1,351	-1H	190,0477	24,48	C7H10O6		HMDB12710	C00944	
3-Deoxy-D-glycero-D-galacto-2-nonulosonic acid	267,07245	1,073	-1H	268,0794	3,08	C9H16O9	Carbohydrates	HMDB00425		5414
3-Indole carboxylic acid glucuronide	338,08722	0,505	+1H	337,0798	5,10	C15H15NO8		HMDB13189		
3-Indolepropionic acid	190,08635	0,500	+1H	189,079	-87,02	C11H11NO2	Indoles and Indole Derivatives	HMDB02302		6602
3-Methyldioxyindole	164,0706	0,015	+1H	163,0633	-3,87	C9H9NO2	Indoles and Indole Derivatives	HMDB04186	C05834	7024
3-O-fucopyranosyl-2-acetamido-2-deoxyglucopyranose	368,15512	0,036	+1H	367,1479	119,10	C14H25NO10	Carbohydrates	HMDB06700		
3-Oxocholic acid	405,26511	1,157	-1H	406,2719	3,19	C24H38O5	Bile Acids	HMDB00502		5488
3-Oxododecanoic acid	213,14966	0,202	-1H	214,1569	-9,89	C12H22O3	Fatty Acids	HMDB10727	C02367	
4-(2-Aminophenyl)-2,4-dioxobutanoic acid	208,06059	0,739	+1H	207,183	55,57	$C_{10}H_9NO_4$	Amino acids and Amino Acid conjugates	HMDB00978	c01252	
4-Hydroxy-3-methylbenzoic acid	151,04036	1,891	-1H	152,0473	6,50	C8H8O3	Aromatic Acids	HMDB04815		
4-Oxoretinal	299,20081	0,877	+1H	298,1933	-2840,84	C20H26O2		HMDB12794		
5,8-Tetradecadienoic acid	223,17067	1,427	-1H	224,339	-2,13	C14H24O2	Fatty Acids	HMDB00560		
5-Acetamidovalerate	158,08241	0,941	-1H	159,0895	44,36	C7H13NO3	Amino acids and Amino Acid conjugates	HMDB12175		
5-Acetylamino-6-formylamino-3-methyluracil	225,06178	5,074	-1H	226,0702	2,56	C8H10N4O4		HMDB11105	c16365	
5-Hydroxy-6-methoxyindole glucuronide	340,10312	1,224	+1H	339,0954	4,28	C15H17NO8	Glucuronides	HMDB10363	C03033	
5-Hydroxyindoleacetic acid	192,06561	0,491	+1H	191,0582	24,45	C10H9NO3	Indoles and Indole Derivatives	HMDB00763	C05635	2975
5-Hydroxymethyl-4-methyluracil	157,06073	0,276	+1H	156,139	8,53	C6H8N2O3	Nucleosides and Nucleoside conjugates	HMDB00544		
7a-Hydroxy-3-oxo-5b-cholanoic acid	391,28482	1,375	+1H	390,277	2,39	C24H38O4	Bile Acids	HMDB00503		5489
9-HODE	295,228	0,455	-1H	296,2351	-12,44	C18H32O3	Fatty Acids	HMDB10223		
Allantoin	157,03693	1,337	-1H	158,044	12,73	C4H6N4O3	Amino Ketones	HMDB00462	C01551	89

all-trans-18-Hydroxyretinoic acid	315,19672	0,428	-1H	316,2039	40,31	C20H28O3		HMDB12452	C16679	
Alpha-Hydroxyhippuric acid	196,06053	0,480	+1H	195,172	693,68	C9H9NO4	Amino acids and Amino Acid conjugates	HMDB02404		
Alpha-ketoisovaleric acid	115,04044	3,270	-1H	116,0473	13,47	C5H8O3	Keto-Acids	HMDB00019	c00141	
Alpha-Linolenic acid	277,2178	1,801	-1H	278,2246	-8,16	C18H30O2	Fatty Acids	HMDB01388	c06427	
Aspartame	295,12906	0,683	+1H	294,1216	4,56	C14H18N2O5	Polypeptides	HMDB01894	C11045	6377
Benzocaine	164,07204	2,022	-1H	165,079	79,57	C9H11NO2		HMDB06044		
Beta-Leucine	130,08762	1,960	-1H	131,0946	3,89	C6H13NO2	Amino acids and Amino Acid conjugates	HMDB03640	C02486	
Calcitroic acid	373,23886	1,176	-1H	374,2457	-3,04	C23H34O4	Alcohols and Polyols	HMDB06472	C18230	
Capric acid	171,13921	0,871	-1H	172,1463	-2,65	C10H20O2	Fatty Acids	HMDB00511	C01571	336
Caproic acid	115,07671	2,210	-1H	116,0837	11,59	C6H12O2	Fatty Acids	HMDB00535	C01585	
Caprylic acid	143,10797	1,465	-1H	144,115	3,31	C8H16O2	Fatty Acids	HMDB00482	C06423	
Carnosol	329,17599	0,502	-1H	330,1831	9,44	C20H26O4	Polyphenols	HMDB02121	C09069	
Cervonoyl ethanolamide	373,27405	0,867	+1H	372,2664	4263,61	C24H36O3		HMDB13627	C13828	
Cervonoyl ethanolamide	373,2738	0,211	+1H	372,2664	21,69	C24H36O3		HMDB13627	C13828	
Cholesterol sulfate	465,3053	1,925	-1H	466,3117	-5,35	C27H46O4S	Steroids and Steroid Derivatives	HMDB00653	c18043	5625
Cholic acid glucuronide	583,31299	1,067	-1H	584,3196	19,52	C30H48O11	Glucuronides	HMDB02577	C03033	6714
Corticosterone	347,22183	0,402	+1H	346,461	16,72	C21H30O4	Cholesterols and derivatives	HMDB01547	c02140	
Creatinine	114,066	1,664	+1H	113,0589	3,36	C4H7N3O	Amino Ketones	HMDB00562	C00791	8
D-Arabitol	151,06136	0,990	-1H	152,0685	5,98	C5H12O5	Alcohols and Polyols	HMDB00568	C00379	
Deoxycholic acid 3-glucuronide	567,31793	0,774	-1H	568,3248	16,37	C30H48O10	Glucuronides	HMDB02596	C03033	6721
Docosahexaenoic acid	327,23343	1,435	-1H	328,2402	-3,01	C22H32O2	Fatty Acids	HMDB02183	co6429	3457
Dodecanoic acid	199,17049	0,672	-1H	200,1776	-2,85	C12H24O2	Fatty Acids	HMDB00638	C02679	5611
dUDP	386,99817	4,766	-1H	388,0073	2,55	C9H14N2O11P2	Nucleotides	HMDB01000	C01346	5931
Ethyladipic acid	175,0965	0,101	+1H	174,0892	21,96	C8H14O4	Dicarboxylic Acids	HMDB02023		6444
Fexofenadine	502,29309	4,166	+1H	501,2879	5,54	C32H39NO4	Diphenylmethanes	HMDB05030	C06999	2766
Formyl-5-hydroxykynurenamine	209,09203	0,208	+1H	208,0848	616,12	C10H12N2O3		HMDB12948	C05647	
Formyl-5-hydroxykynurenamine	207,07764	0,575	-1H	208,0848	-212,76	C10H12N2O3		HMDB12948	C05647	
Gamma-Glutamyltyrosine	311,1239	0,449	+1H	310,1165	9,59	C14H18N2O6		HMDB11741		
Gamma-Linolenic acid	277,21762	1,142	-1H	278,2246	-12,41	C18H30O2	Fatty Acids	HMDB03073	C06426	386
Hexanoylglycine	174,11247	0,015	+1H	173,21	289,69	C8H15NO3	Amino acids and Amino Acid conjugates	HMDB00701	C02710	
Hippuric acid	178,05125	1,604	-1H	179,0582	9,12	C9H9NO3	Amino acids and Amino Acid conjugates	HMDB00714	c01586	
Hippuric acid	180,06546	0,328	+1H	179,173	6,74	C9H9NO3	Amino acids and Amino Acid conjugates	HMDB12884	c01586	
Homovanillic acid sulfate	261,00754	0,400	-1H	262,0147	134,19	C9H10O7S		HMDB11719	C05582	
Hyodeoxycholic acid	391,28589	1,244	-1H	392,572	-3,03	C24H40O4	Bile Acids	HMDB00733		
Imidazolelactic acid	155,04655	2,138	-1H	156,0535	10,72	C6H8N2O3	Hydroxy Acids	HMDB02320	C05132	6617
Indole-3-carbinol	148,07552	1,226	+1H	147,0684	792,97	C9H9NO	Indoles and Indole Derivatives	HMDB05785		
Indoleacrylic acid	186,05629	1,292	-1H	187,0633	208,72	C11H9NO2	Indoles and Indole	HMDB00734	C00331	5702

							Derivatives			
Indoleacrylic acid	188,07069	0,502	+1H	187,063 3	110,10	C11H9NO2	Indoles and Indole Derivatives	HMDB00734	C00331	5702
Indolelactic acid	206,08124	0,311	+1H	205,21	13,51	C11H11NO3	Indoles and Indole Derivatives	HMDB00671		
Indolelactic acid	204,06674	0,585	-1H	205,073 9	3,06	C11H11NO3	Indoles and Indole Derivatives	HMDB00671	C02043	
Indoxyl	134,05984	1,473	+1H	133,052 8	-45,02	C8H7NO	Indoles and Indole Derivatives	HMDB04094	C05658	7014
Isobutyryl-L-carnitine	232,15448	0,606	+1H	231,289	7,96	C11H21NO4	Amino acids and Amino Acid conjugates	HMDB00736		
Isocitric acid	191,0199	0,875	-1H	192,027	5,22	C6H8O7	Tricarboxylic Acids	HMDB00193	C00311	
Isovalerylcarnitine	246,1701	0,510	+1H	245,315	4,44	C12H23NO4	Amino acids and Amino Acid conjugates	HMDB00688		
Isovalerylglycine	160,09674	0,467	+1H	159,183	25,49	C7H13NO3	Amino acids and Amino Acid conjugates	HMDB00678		
Kynurenic acid	190,04973	0,712	+1H	189,042 6	1422,36	C10H7NO3	Aromatic Acids	HMDB00715	C01717	5683
Lanthionine ketimine	188,00232	0,065	-1H	189,009 6	6,97	C6H7NO4S	Amino Acids	HMDB04823		7085
Leukotriene B5	335,22278	3,247	+1H	334,214 4	53,95	C20H30O4	Eicosanoids	HMDB05073		
L-Gulose + 13 altri metaboliti ad uguale delta	179,05638	1,512	-1H	180,063 4	2,48	C6H12O6	Carbohydrates	HMDB12326	C15923	
L-phenylalanyl-L-hydroxyproline	279,13431	1,380	+1H	278,126 6	36,14	C14H18N2O 4	Polypeptides	HMDB11176		
LysoPC(14:0)	468,30832	0,289	+1H	467,301 2	only CTR	C22H46NO7 P	Phospholipids	HMDB10379	C04230	
LysoPC(16:0)	496,33948	0,580	+1H	495,332 5	-32,69	C24H50NO7 P	Phospholipids	HMDB10382	C04230	
LysoPC(16:1(9Z))	494,32425	0,284	+1H	493,316 8	only CTR	C24H48NO7 P	Phospholipids	HMDB10383	C04230	
LysoPC(17:0)	510,35513	0,565	+1H	509,348 1	-5,47	C25H52NO7 P	Phospholipids	HMDB12108	C04230	
lysoPC(18:2(9Z,12Z))	520,34033	1,057	+1H	519,332 5	-2,71	C26H50NO7 P	Phospholipids	HMDB10386	C04230	
LysoPC(18:2(9Z,12Z))	520,33984	0,117	+1H	519,651	-2,05	C26H50NO7 P	Phospholipids	HMDB10386	C04230	
LysoPC(18:3(9Z,12Z,15Z))	518,32416	0,118	+1H	517,316 8	only CTR	C26H48NO7 P	Phospholipids	HMDB10388	C04230	
LysoPC(18:4(6Z,9Z,12Z,15Z))	520,33881	4,454	+1H	515,62	-2,35	C26H46NO7 P	Phospholipids	HMDB10389	C04230	
lysoPC(20:2(11Z,14Z))	548,37061	0,780	+1H	547,677	3,07	C28H54NO7 P	Phospholipids	HMBD10392	C04230	
lysoPC(20:4(8Z,11Z,14Z,17Z))	544,33997	0,337	+1H	543,332 5	-5,08	C28H50NO7 P	Phospholipids	HMDB10396	C04230	
lysoPC(20:5(5Z,8Z,11Z,14Z,17Z))	542,32416	1,127	+1H	541,316 8	only CTR	C28H48NO7 P	Phospholipids	HMDB10397	C04230	
LysoPC(22:5(7Z,10Z,13Z,16Z,19Z))	570,35492	0,857	+1H	569,348 1	only CTR	C30H52NO7 P	Phospholipids	HMDB10403	C04230	
lysoPC(22:6(4Z,7Z,10Z,13Z,16Z,19Z))	404,20688	0,215	+1H	567,332 5	-5,61	C30H50NO7 P	Phospholipids	HMDB10404	C04230	
LysoPE(16:0/0:0)	452,27896	1,509	-1H	453,285 6	-2,43	C21H44NO7 P	Phospholipids	HMDB11503		
LysoPE(18:0/0:0)	482,32407	0,089	+1H	481,316 8	only CTR	C23H48NO7 P	Phospholipids	HMDB11130		
LysoPE(18:1(9Z)/0:0)	478,29483	1,937	-1H	479,301 2	-2,60	C23H46NO7 P	Phospholipids	HMDB11506		
LysoPE(18:2(9Z,12Z)/0:0)	478,29269	0,256	+1H	477,285 6	-2,41	C23H44NO7 P	Phospholipids	HMDB11507		
LysoPE(18:2(9Z,12Z)/0:0)	476,27878	1,022	-1H	477,285 6	-4,78	C23H44NO7 P	Phospholipids	HMDB11507		
LysoPE(18:2(9Z,12Z)/0:0)	478,29297	0,321	+1H	477,285 6	4,71	C23H44NO7 P	Phospholipids	HMDB11507		
LysoPE(18:2(9Z,12Z)/0:0)	476,2789	1,278	-1H	477,285 6	-2,06	C23H44NO7 P	Phospholipids	HMDB11507		
LysoPE(20:1(11Z)/0:0)	508,33951	0,506	+1H	507,332 5	-47,70	C25H50NO7 P	Phospholipids	HMDB11512		
LysoPE(22:6(4Z,7Z,10Z,13Z,16Z,19Z)/0:0)	526,29181	1,006	+1H	525,285 5	43,51	C27H44NO7 P	Phospholipids	HMDB11526		

Methyldopa	210,07732	0,641	-1H	211,0845	-24,18	C10H13NO4		HMDB11754	C07194	
MG(14:1(9Z)/0:0/0:0)	299,22336	1,872	-1H	300,2301	-2,94	C17H32O4	Glycerolipids	HMDB11562		
Monoethylhexyl phthalic acid	279,15933	0,943	+1H	278,1518	9,26	C16H22O4		HMDB13248	C03343	
Myristic acid	227,20198	1,472	-1H	228,371	-5,51	C14H28O2	Fatty Acids	HMDB00806	c06424	
N-Acetyl-4-O-acetylneuraminic acid	350,10941	0,383	-1H	351,1165	13,46	C13H21NO10	Carbohydrates	HMDB00796	C04015	5761
N-Acetylaspartylglutamic acid	303,08353	0,443	-1H	304,0907	2,14	C11H16N2O8	Polypeptides	HMDB01067	C12270	
N-Acetylserine	146,04599	0,728	-1H	147,0532	14,91	C5H9NO4	Amino acids and Amino Acid conjugates	HMDB02931		
Nicotinuric acid	181,06079	0,100	+1H	180,0535	227,77	C8H8N2O3	Cyclic Amines	HMDB03269	C05380	1499
N-Methylnicotinamide	137,07057	2,676	+1H	136,0637	23,49	C7H8N2O	Cyclic Amines	HMDB03152		
Norsalsolinol	164,07188	1,097	-1H	165,079	-52,54	C9H11NO2		HMDB06044		
Nutriacholic acid	389,26981	0,189	-1H	390,277	10,69	C24H38O4	Bile Acids	HMDB00467		
Octanoylglucuronide	319,14005	0,613	-1H	320,1471	72,05	C14H24O8	Glucuronides	HMDB10347	C03033	
o-Tyrosine	180,06668	0,325	-1H	181,0739	-16,28	C9H11NO3	Amino Acids	HMDB06050		
Palmitelaidic acid	253,21761	1,190	-1H	254,2246	-4,47	C16H30O2	Fatty Acids	HMDB12328		
PE(P-16:0e/0:0)	438,29764	0,588	+1H	437,2906	3,72	C21H44NO6P		HMDB11152		
Pentadecanoic acid	241,21758	1,123	-1H	242,2246	-6,58	C15H30O2	Fatty Acids	HMDB00826	c16537	5789
Phenylacetylglycine	194,08133	0,805	+1H	193,199	4042,09	C10H11NO3	Amino acids and Amino Acid conjugates	HMDB00821	c05596	
PI(18:1(9Z)/16:0)	835,53534	1,329	-1H	836,5415	-2,70	C43H81O13P	Phospholipids	HMDB09834	C00626	
PS(22:6(4Z,7Z,10Z,13Z,16Z,19Z)/18:2(9Z,12Z))	830,49829	0,660	-1H	831,505	6,35	C46H74NO10P	Phospholipids	HMDB12446		
Pseudouridine	243,06235	0,366	-1H	244,0695	13,82	C9H12N2O6	Nucleoside Analogues	HMDB00767	C02067	5734
Pteroyltriglutamic acid	698,21515	1,572	-1H	699,625	1033,75	C29H33N9O12	Heterocyclic molecules	HMDB01902		
Retinyl ester	301,21774	1,456	-1H	302,2246	-3,80	C20H30O2	Retinoids	HMDB03598	C02075	6966
Riboflavin	377,14581	0,697	+1H	376,1383	12,76	C17H20N4O6	Pterins	HMDB00244	C00255	5249
Sphingosine 1-phosphate	380,25635	0,851	+1H	379,2487	-1648,66	C18H38NO5P	Sphingolipids	HMDB00277	C06124	5272
Suberic acid	173,08203	0,599	-1H	174,0892	249,43	C8H14O4	Dicarboxylic Acids	HMDB00893	c08278	
Suberic acid	175,09656	0,452	+1H	174,194	24,23	C8H14O4	Dicarboxylic Acids	HMDB00893	c08278	
Succinyladenosine	384,11508	0,206	+1H	383,1077	246,66	C14H17N5O8	Nucleoside Analogues	HMDB00912		5867
Sulfolithocholylglycine	512,26959	1,690	-1H	513,276	5,72	C26H43NO7S	Acyl Glycines	HMDB02639	C11301	6725
Sulfolithocholylglycine	514,28284	0,857	+1H	513,276	5610,52	C26H43NO7S	Acyl Glycines	HMDB02639	C11301	6725
Taurochenodesoxycholic acid	500,30405	0,035	+1H	499,2968	7,26	C26H45NO6S	Bile Acids	HMDB00951	C05465	5897
Taurocholic acid	514,28485	0,854	-1H	515,2917	13,45	C26H45NO7S	Bile Acids	HMDB00036	C15516	
Taurodeoxycholic acid	498,28973	0,514	-1H	499,2968	12,95	C26H45NO6S	Bile Acids	HMDB00896	C05465	
Tetracosahexaenoic acid	357,27887	0,221	+1H	356,2715	-5,28	C24H36O2	Fatty Acids	HMDB02007		6430
trans-2-Octenoic acid	143,10652	0,946	+1H	142,0994	3,35	C8H14O2	Fatty Acids	HMDB01568		6328
trans-3-Hydroxycotinine glucuronide	369,13	2,037	+1H	368,122	2237,58	C16H20N2O8	Glucuronides	HMDB01204	C03033	6080
trans-Tetra-dec-2-enoic acid	225,18633	1,406	-1H	226,1933	-4,31	C14H26O2	Fatty Acids	HMDB10732		
Traumatic acid	227,12912	1,060	-1H	228,1362	-3,21	C12H20O4	Dicarboxylic Acids	HMDB00933	C16308	5882
Uric acid	167,02132	1,527	-1H	168,0283	17,81	C5H4N4O3	Purines and Purine	HMDB00289	C00366	88

							Derivatives			
Valerylcarnitine	246,16989	0,362	+1H	245,1627	5,73	C12H23NO4		HMDB13128		
* fold change 2h post-resuscitation after cardiac arrest vs control										

Bold metabolites: metabolites whose identification was based on MSn spectra matching with authentic standard present in HMBD metabolomic database

Supplemental Table 4. Metabolic pathway analysis by MetaboAnalyst's tools

	Total	Hits	Raw p	negLog(p)	Holm adjust	FDR	Impact
Tryptophan metabolism	79	8	0,0002883	8,1517	0,02306	0,02306	0,02286
Fatty acid biosynthesis	49	4	0,022723	3,7844	1	0,90891	0
Retinol metabolism	22	2	0,08576	2,4562	1	1	0
Sphingolipid metabolism	25	2	0,10679	2,2369	1	1	0,04292
Valine, leucine and isoleucine degradation	40	2	0,22572	1,4885	1	1	0,02803
Primary bile acid biosynthesis	47	2	0,28457	1,2568	1	1	0,01838
Linoleic acid metabolism	15	1	0,28919	1,2407	1	1	0
Pentose and glucuronate interconversions	53	2	0,33484	1,0941	1	1	0,009
Taurine and hypotaurine metabolism	20	1	0,36595	1,0053	1	1	0
Citrate cycle (TCA cycle)	20	1	0,36595	1,0053	1	1	0,05826
Riboflavin metabolism	21	1	0,38029	0,96682	1	1	0,14504
Pantothenate and CoA biosynthesis	27	1	0,4599	0,77674	1	1	0,07366
Valine, leucine and isoleucine biosynthesis	27	1	0,4599	0,77674	1	1	0,0885
Phenylalanine, tyrosine and tryptophan biosynthesis	27	1	0,4599	0,77674	1	1	0,09102
alpha-Linolenic acid metabolism	29	1	0,48414	0,72539	1	1	0,20335
Tyrosine metabolism	76	2	0,51423	0,66508	1	1	0,05093
Vitamin B6 metabolism	32	1	0,5185	0,65681	1	1	0,02697
Glycerophospholipid metabolism	39	1	0,59019	0,52732	1	1	0,00317
Inositol phosphate metabolism	39	1	0,59019	0,52732	1	1	0,0499
Nicotinate and nicotinamide metabolism	44	1	0,63487	0,45434	1	1	0
Phenylalanine metabolism	45	1	0,64321	0,44128	1	1	0,0315
Steroid hormone biosynthesis	99	2	0,65998	0,41554	1	1	0,0457
Starch and sucrose metabolism	50	1	0,6822	0,38243	1	1	0,01265
Glyoxylate and dicarboxylate metabolism	50	1	0,6822	0,38243	1	1	0,02494
Pyrimidine metabolism	60	1	0,74806	0,29027	1	1	0
Arginine and proline metabolism	77	1	0,83063	0,18558	1	1	0,00645
Purine metabolism	92	1	0,88098	0,12672	1	1	0,00969

Total: total number of compounds in the pathway
Hits: matched number of metabolites from the uploaded list
Raw p: p-value from enrichemnt analysis
Holm adjust: p value adjusted by Holm-Bonferroni method
FDR: p value adjusted using False Discovery Rate
Impact: pathway impact calculated from topology analysis

ACKNOWLEDGEMENTS

Publications derived from the thesis work

Full articles:

- Ristagno G, Fries M, Brunelli L, Fumagalli F, Bagnati R, Russo I, Staszewsky L, Masson S, Volti GL, Zappalà A, Derwall M, Brücken A, Pastorelli R, Latini R. Early kynurenine pathway activation following cardiac arrest in rats, pigs, and humans. Resuscitation 2013; Nov;84(11):1604-10
- Giuseppe Ristagno, Yongqin Li, Francesca Fumagalli, Andrea Finzi, Weilun Quan. Amplitude spectrum area to guide resuscitation - A retrospective analysis during out-of-hospital cardiopulmonary resuscitation in 609 patients with ventricular fibrillation cardiac arrest. Resuscitation 2013; Dec;84(12):1697-703
- Scapigliati A, Ristagno G, Cavaliere F. The Best Timing For Defibrillation In Shockable Cardiac Arrest. Minerva Anestesiol. 2013 Jan;79(1):92-101 .
- Laura Brunelli, Giuseppe Ristagno, Renzo Bagnati, Francesca Fumagalli, Roberto Latini, Roberto Fanelli, Roberta Pastorelli. A combination of untargeted and targeted metabolomics approaches unveils changes in the kynurenine pathway following cardiopulmonary resuscitation. Metabolomics 2013 in press [Epub ahead of print].

Abstracts:

- Ristagno G, Tan Q, Quan W, Freeman G, Bisera J, Tang W. AMSA-based shock decision: a human retrospective analyses during pre-Hospital CPR intervention. Circulation 2010; 122:A2054
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- Russo I, Staszewsky L, Fumagalli F, Masson S, Latini R, Ristagno G. Temporal Relationship Between Left Ventricular Dysfunction and Plasma High-Sensitivity Cardiac Troponin T Levels in a Rat Model of Cardiac Arrest and Resuscitation. Circulation 2011; 124: A200.
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- Ristagno G, Quan W, Freeman G. Amplitude spectrum area to predict defibrillation outcome after recurrent and defibrillation resistant ventricular fibrillation during pre-hospital cardiopulmonary resuscitation. Resuscitation 2012;e11-12.
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- Ristagno G, Brunelli L, Fries M, Fumagalli F, Bagnati, Pastorelli R, Latini R. Kynurenine Pathway Activation Following Resuscitation from Cardiac Arrest: An Experimental and Clinical Investigation---from a Rat and a Pig Model to a Preliminary Clinical Validation. Circulation 2012;126:A223.
- Ristagno G, Fornari C, Li Y, Fumagalli F, Finzi A, Mauri T, Rossi G, Latini R, Cesana G, Pesenti A. Amplitude Spectrum Area-Based Defibrillation Decision During Prehospital Cardiopulmonary Resuscitation in Lombardia, Italy. Circulation 2012;126:A14.

- Ristagno G, Quan W, Freeman G. Amplitude Spectrum Area Based Defibrillation Decision Greatly Improves Shock Success Rate and Accuracy During Prehospital Cardiopulmonary Resuscitation. Circulation 2012;126:A224.

Congress presentations related to the thesis work

- "Amplitude Spectrum Area (AMSA) as predictor of successful defibrillation", in "23nd Annual Meeting, A.P.I.C.E. 2010", 5-11 November 2010, Catania, Italy.
- "La defibrillazione: i defibrillatori di nuova generazione, le forme d'onda, le energie, l'algoritmo di trattamento" in the National Congress "Metodologia e contenuti delle Linee Guida 2010 dell'arresto cardiaco e delle emergenze cardio-circolatorie. Quarto D'Altino 11 February 2011, Italy.
- "Future developments of AED programmes: are going to face a CPR revolution?" nella sessione "Hot topics in Basic Life Support and Early Defibrillation" in the National Congress of the Italian Resuscitation Council 2011. Bologna, 28-30 April 2011, Italy.
- "Predizione del successo della defibrillazione con i nuovi DAE e implicazioni nella strategia di defibrillazione" in the session "Emergenza" in the 22° Congresso SMART, Milan, 25-27 May 2011, Italy.
- "Biomarkers during CPR", 4th Guangzhou Conference on CPR, Sun Yat-sen Memorial University, June 22-24 2012, Guangzhou, China.
- "Priorities of intervention and predictors of success of defibrillation" in the International congress "Weil Conference – conference on cardiac arrest, shock and trauma", 8-9 September 2012, Istituto Mario Negri, Milan, Italy.
- "Sindrome post-rianimazione e sepsi: quali affinità?" in the session "La Sindrome Post-Rianimazione" at the "66° Congresso Nazionale" della Società Italiana Anestesia Analgesia Rianimazione Terapia Intensiva, Napoli 24-27 October 2012, Italy.
- "Nuove Strategie di defibrillazione", XXV National congress of the Società Italiana di Terapia Intensiva, SITI, Università Cattolica del Sacro Cuore, Roma, 9-10 November 2012, Italy.

- "I defibrillatori e il successo nella defibrillazione" in the National congress "Hypothermia 2012. The cardiac arrest and post resuscitation care", 15-16 November, Genova, Italy.
- "Looking for Biomarkers Predictive of Outcome", in the session "Cardiac Arrest Part I: Advanced Research" in "APICE Masterclass 2012 – 25th Annual International Meeting", Catania, 30 November – 2 December 2012, Italy.
- "Fisiopatologia della sindrome post arresto cardiaco", in the session "La sindrome post arresto cardiaco", in " 1° Congresso Nazionale di Ipotermia Terapeutica in Cardiologia – Una nuova era nelle cure post-arresto cardiaco", Padova, 1 February 2013, Italy.
- "Nuovi biomarcatori circolanti predittivi di outcome dopo rianimazione da arresto cardiaco" in the session "Emergenza I", 24° Congresso SMART, Milano, 8-10 May 2013, Italy.
- "Fisiopatologia della sindrome post-arresto cardiaco", in the session "La sindrome post-arresto cardiaco" in "Gli Aggiornamenti della Scuola in tema di: Sindrome post-arresto cardiaco, Aritmologia interventistica, Supporti meccanici all'assistenza ventricolare, Risonanza magnetica e diagnostica cardiologica, Ischemia miocardica. Scuola di Specializzazione in Malattie dell'Apparato Cardiovascolare, Università degli Studi di Palermo, Palermo 17-18 May 2013, Italy.
- "AMSA-based shock decision: a human retrospective analyses during pre-Hospital CPR intervention", American Heart Association "Scientific Sessions" annual congress, 13-17 November 2010, Chicago, Illinois, USA. (*oral presentation*)
- "AMSA for monitoring depth of chest compression during out-of-hospital cardiopulmonary resuscitation", European Resuscitation Council 2010 congress, 2-4 December 2010, Porto, Portugal. (*oral presentation*)

- "Improved accuracy of prediction of defibrillation success by shortening amplitude spectrum area integration intervals", European Resuscitation Council annual meeting, 14-15 October 2011Valletta, Malta. (*poster presentation*).
- "Mechanisms involved in post resuscitation myocardial dysfunction in a rat model of cardiac arrest and resuscitation" in the session "Free papers 1", European Resuscitation Council annual meeting, 14-15 October 2011Valletta, Malta. (*oral presentation*).
- "Significant decreases in amplitude spectrum area during pre-defibrillation pauses in chest compression in out-of-hospital cardiac arrest patients" in the session "Young Investigators Competition", European Resuscitation Council annual meeting, 14-15 October 2011Valletta, Malta. (*oral presentation*).
- "Temporal Relationship Between Left Ventricular Dysfunction and Plasma High-Sensitivity Cardiac Troponin T Levels in a Rat Model of Cardiac Arrest and Resuscitation", American Heart Association "Resuscitation Science Symposium", 12-15 November 2011, Orlando, Florida, USA. (*poster presentation*).
- "Specific Metabolic Pathways Involved in Outcome of Cardiopulmonary Resuscitation: A Pilot Plasma Metabolomic Study in a Rat Model of Cardiac Arrest and CPR", American Heart Association "Resuscitation Science Symposium", 12-15 November 2011, Orlando, Florida, USA. (*poster presentation*).
- "Amplitude spectrum area to predict defibrillation outcome after recurrent and defibrillation resistant ventricular fibrillation during pre-hospital cardiopulmonary resuscitation" in the session "Free paper", European Resuscitation Council annual meeting, 18-20 October 2012 Vienna, Austria. *(oral presentation).*
- "Rapid decreases in Amplitude Spectrum Area after interruption in chest compression in out-of-hospital cardiac arrest patients" in the session "Free paper",

European Resuscitation Council annual meeting, 18-20 October 2012 Vienna, Austria. *(oral presentation).*

- "Kynurenine Pathway Activation Following Resuscitation from Cardiac Arrest: An Experimental and Clinical Investigation---from a Rat and a Pig Model to a Preliminary Clinical Validation", American Heart Association "Resuscitation Science Symposium", 3-4 November 2012, Los Angeles, California, USA. *(poster presentation).*
- "Amplitude Spectrum Area-Based Defibrillation Decision During Prehospital Cardiopulmonary Resuscitation in Lombardia, Italy", American Heart Association "Resuscitation Science Symposium", 3-4 November 2012, Los Angeles, California, USA. *(poster presentation).*
- "Amplitude Spectrum Area Based Defibrillation Decision Greatly Improves Shock Success Rate and Accuracy During Prehospital Cardiopulmonary Resuscitation", American Heart Association "Resuscitation Science Symposium", 3-4 November 2012, Los Angeles, California, USA. *(poster presentation).*

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Experimental paper

Early kynurenine pathway activation following cardiac arrest in rats, pigs, and humans☆

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ABSTRACT

Aim of the study: Kynurenine pathway (KP) is a major route of the tryptophan (TRP) catabolism. In the present study, TRP and KP metabolites concentrations were measured in plasma from rats, pigs and humans after cardiac arrest (CA) in order to assess KP activation and its potential role in post-resuscitation outcome.

Methods: Plasma was obtained from: (A) 24 rats, subjected to 6 min CA and 6 min of cardiopulmonary resuscitation (CPR); (B) 10 pigs, subjected to 10 min CA and 5 min CPR; and (C) 3 healthy human volunteers and 5 patients resuscitated from CA. KP metabolites were quantified by liquid chromatography multiple reaction monitoring mass spectrometry. Assessments were available at baseline, and 1–4 h, and 3–5 days post-CA.

Results: KP was activated after CA in rats, pigs, and humans. Decreases in TRP occurred during the post-resuscitation period and were accompanied by significant increases in its major metabolites, 3-hydroxyanthranilic acid (3-HAA) and kynurenic acid in each species, that persisted up to 3–5 days post-CA ($p < 0.01$). In rats, changes in KP metabolites reflected changes in post-resuscitation myocardial function. In pigs, changes in TRP and increases in 3-HAA were significanlty related to the severity of cerebral histopathogical injuries. In humans, KP activation was observed, together with systemic inflammation. Post-CA increases in 3-HAA were greater in patients that did not survive.

Conclusion: In this fully translational investigation, the KP was activated early following resuscitation from CA in rats, pigs, and humans, and might have contributed to post-resuscitation outcome.

1. Introduction

After the initial success of cardiopulmonary resuscitation (CPR), the majority of resuscitated patients die within 72 h, due to what is now termed "post-cardiac arrest syndrome".[1–3] Most prominent are post-resuscitation myocardial failure, ischemic brain damage and processes related to the systemic ischemia/reperfusion response.[3–8]

Due to the complexity and interplay of events occurring during the post-cardiac arrest syndrome,[3,9] predicting survival and functional outcome in patients resuscitated from cardiac arrest remains a difficult task, especially in the early post-resuscitation phase.[10,11] In earlier investigations in a rat model of cardiac arrest and CPR, we adopted an untargeted metabolomics approach to identify perturbations in post-resuscitation circulating metabolites.[12] Following resuscitation, we identified alterations in a major route of the tryptophan (TRP) catabolism, namely kynurenine pathway (KP, Fig. 1). KP is mainly activated upon inflammatory stimulation and is implicated in the pathogenesis of numerous central nervous system disorders, as well as in sepsis development and profound hypotension during septic shock.[13,14] KP activation has been also described in various clinical conditions, including infection, autoimmune syndromes, malignancies, depression, and pregnancy.[15,16]

☆ A Spanish translated version of the abstract of this article appears as Appendix in the final online version at http://dx.doi.org/10.1016/j.resuscitation.2013.06.002.

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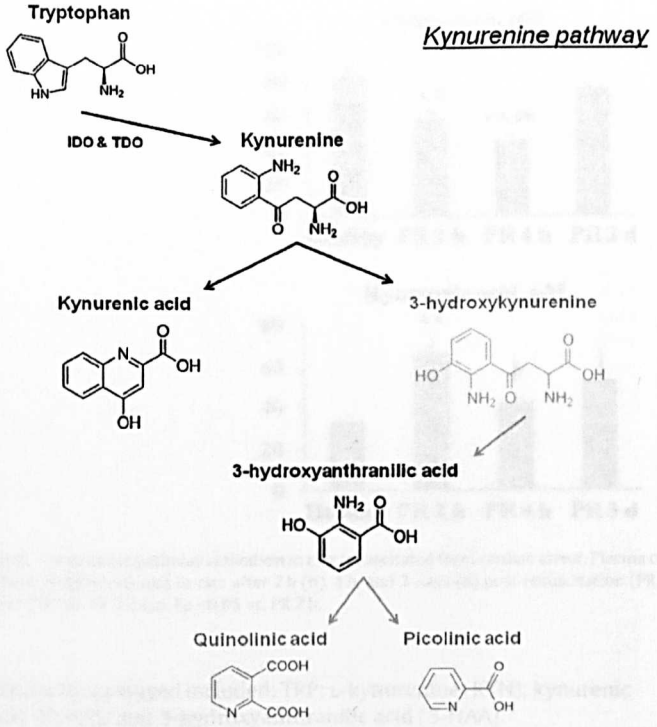

Fig. 1. Tryptophan degradation through the kynurenine pathway. In black are the metabolites assayed in the present study. TDO, tryptophan 2,3-dioxygenase; IDO, indoleamine 2,3-dioxygenase.

The present study aimed to investigate KP activation after cardiac arrest and its relationship with the severity of post-cardiac arrest syndrome, by a fully translational approach. More specifically, KP was assessed during the initial hours and days following resuscitation from cardiac arrest in rats, pigs, and humans. We hypothesized that the KP would be activated following cardiac arrest and this activation would be associated with the severity of post-resuscitation organ dysfunctions and outcome.

2. Methods

Detailed methods on experimental procedures, clinical studies, and metabolite measurements, are reported in the "Supplementary Material".

Procedures involving animals and their care were in compliance with national (D.L. n. 116, G.U., suppl. 40, 18 February 1992, Circolare no. 8, G.U., 14 Luglio 1994) and international laws and policies (EEC Council Directive 86/609, OJL 358, 1, December 12, 1987; Guide for the Care and Use of Laboratory Animals, US National Research Council, 1996). Approvals of the studies were obtained by the local institutional review board committees and governmental institutions. KP activation was initially investigated in rats, at the Mario Negri Institute, Milan, Italy. Results were then validated in plasma obtained from earlier experiments in pigs performed at Aachen University.[17] Clinical validation of animal results was further performed in plasma obtained from 3 healthy volunteers and 5 patients resuscitated from cardiac arrest of non-traumatic origin. These patients were prospectively studied in another trial that has been previously published and in which the influence of therapeutic hypothermia on S-100B values after cardiac arrest has been studied.[18,19]

2.1. Experimental models

2.1.1. Rats

2.1.1.1. Animal preparation. Twenty four male Sprague–Dawley rats were used for the study. Animals were anesthetized and instrumented for hemodynamic measurements and induction of cardiac arrest, as previously described.[20]

2.1.1.2. Experimental procedures. An established model of electrically induced cardiac arrest and CPR has been used.[20] Briefly, animals were subjected to 6 min of untreated cardiac arrest and 6 min of mechanical chest compression and ventilations prior to defibrillation. After resuscitation, rats were sacrificed at different time points: 2 h post-resuscitation ($n=6$); 4 h post-resuscitation ($n=6$); and 3 days post-resuscitation ($n=5$). Additional rats were sacrificed at baseline, prior to inducing cardiac arrest, and served as healthy rats ($n=7$).

2.1.1.3. Measurements. Hemodynamics and echocardiography were performed as described previously.[20,21] Plasma high sensitivity cardiac troponin T (hs-cTnT) concentration was assessed with an electrochemiluminescence assay (ECLIA, Elecsys 2010 analyzer, Roche Diagnostics, Germany). Cardiac isoprostanes were assessed by ELISA method.

2.1.2. Pigs

2.1.2.1. Animal preparation. Ten male domestic pigs were used for this study. Pigs were anesthetized and surgically prepared as previously described.[17]

2.1.2.2. Experimental procedures. An established model of electrically induced cardiac arrest and CPR has been used.[17] Briefly, animals were subjected to 10 min of untreated cardiac arrest and 5 mins of mechanical chest compression and ventilations prior to defibrillation. After resuscitation, pigs were observed up to 5 days post-resuscitation.

2.1.2.3. Measurements. Hemodynamics, neurological recovery, and cerebral histology were measured and recorded as described previously.[17]

2.2. Human studies

2.2.1. Patients

Five adult non traumatic out-of-hospital cardiac arrest patients were included. CPR and post-resuscitation treatments were based on standardized protocols, as previously reported.[18,19] Among the 5 cardiac arrest patients included in the study, 3 received hypothermia treatment, while 2 did not. Three healthy volunteers served as control for plasma KP metabolites.

2.2.2. Data collection

Clinical data were collected 1 h after intensive care unit (ICU) admission and 3 days later, using a web-based data entry system complying with the Utstein-Style.[18,19] Blood samples for the determination of C-reactive protein (CRP), procalcitonin (PCT), tumor necrosis factor alpha (TNF-a), interleukin 6 and 8 (IL-6, IL-8), macrophage inhibitor factor (MIF), and KP metabolites, were taken at the same time points.

2.3. TRP and KP metabolites measurements

Plasma samples (20 μL) from rats, pigs and humans were used. Absolute quantification of plasma TRP and KP metabolites was performed by liquid chromatography-multiple reaction monitoring coupled with isotope-dilution mass spectrometry (LC–MRM-MS).

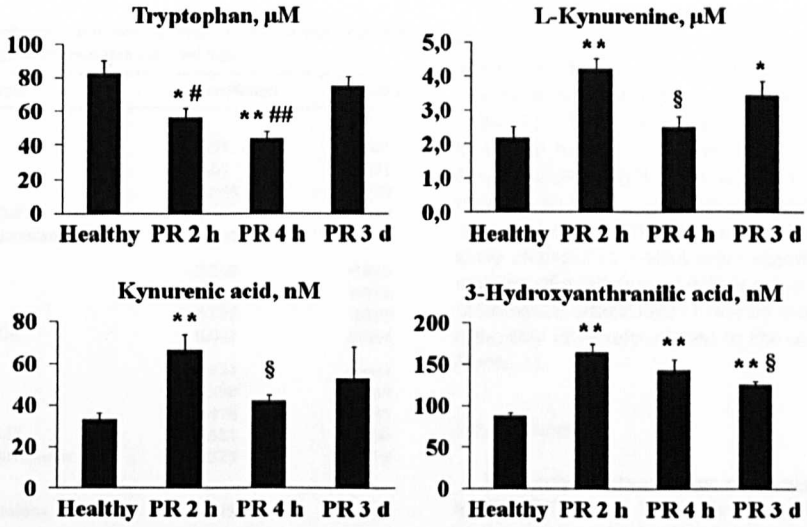

Fig. 2. Kynurenine pathway activation in rats resuscitated from cardiac arrest. Plasma concentrations of trypthophan, L-kynurenine, kynurenic acid and 3-hydroxyanthranilic acid in healthy rats and in rats after 2 h (h), 4 h, and 3 days (d) post-resuscitation (PR). Data are reported as mean ± SEM. $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. healthy; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ vs. PR 3 days; $^{\S}p < 0.05$ vs. PR 2 h.

Metabolites assayed included: TRP; L-kynurenine (KYN); kynurenic acid (KYNA); and 3-hydroxyanthranilic acid (3-HAA).

2.4. Statistical analysis

Normal distribution of the data was confirmed using the one sample Kolmogorov–Smirnov *Z* test. For comparisons among time-based measurements within groups one-way ANOVA with Tukey Kramer's multiple comparisons was used. Linear correlations were calculated using the Pearson correlation coefficient. All data are reported as mean ± SEM. A 2-tail $p < 0.05$ was considered as statistically significant. All analyses were performed by SPSS 16 (SPSS Inc, Chicago, IL).

3. Results

3.1. Rats

At 2 and 4 h post-resuscitation resuscitation, plasma levels of TRP were significantly lower in cardiac arrest animals compared to

Table 1
Hemodynamics, functional and histological outcome, and biomarkers in resuscitated rats and pigs.

Rats	Healthy	PR 2 h	PR 4 h	PR 3 days
MAP, mmHg	136 ± 2	105 ± 2**	105 ± 5**	N.A.
CPP, mmHg	116 ± 3	87 ± 2**	91 ± 4**	N.A.
EF, %	79 ± 2	42 ± 5**	48 ± 6**	75 ± 3##§§
EDV, µL	378 ± 24	446 ± 51	465 ± 57	280 ± 22*#§
SV, mL	0.34 ± 0.02	0.21 ± 0.02**	0.18 ± 0.02**	0.22 ± 0.02**
DT, msec	28.0 ± 3.9	18.8 ± 1.2	19.4 ± 1.3	27.6 ± 2.8#§
hs-cTnT, ng/L	57 ± 18	4453 ± 615**	3874 ± 935**	669 ± 531##§
c-Isoprostanes, pg/µg prot	7 ± 2.2	57 ± 8**	47 ± 5**	14 ± 3.2##§§

Pigs	Baseline	PR 1 h	PR 4 h
MAP, mmHg	121 ± 4	76 ± 5**	86 ± 4**
CO, mL/min	5.8 ± 0.3	2.9 ± 0.3**	3.4 ± 0.2**

Pigs	PR 1 day (score)	PR 3 days (score)	PR 5 days (score)
NDS	66.5 ± 5.3	39.5 ± 10.3	41 ± 12.6
Brain lesion			
Cortex			7.7 ± 1
CA1			6.5 ± 0.7
Brain necrosis			
Cortex			1.5 ± 0.2
CA1			2.4 ± 0.4
Brain astrogliosis			
Cortex			1.4 ± 0.2
CA1			1.8 ± 0.25

MAP, mean arterial pressure; CPP, coronary perfusion pressure; EF, ejection fraction; SV, stroke volume; DT, deceleration time; EDV, end diastolic volume; hs-cTnT, high sensitive cardiac troponin T; c-Isoprostanes, cardiac isoprostane; CO, cardiac output; NDS, neurological deficit score; CA1, CA1 hippocampal sector; PR, post-resuscitation; N.A., not available.
$^{**}p < 0.01$ and $^{*}p < 0.05$ vs. Healthy or Baseline; $^{\#\#}p < 0.01$ and $^{\#}p < 0.05$ vs. PR 2 h; $^{\S\S}p < 0.01$ and $^{\S}p < 0.05$ vs. PR 4 h.

Table 2
Correlation between KP metabolites and hemodynamics, myocardial function, biomarkers, and brain histology in resuscitated rats and pigs.

KP metabolite	Variable	r coefficient	p value
Rats			
TRP	EF	0.55	0.005
	SV	0.53	0.011
	DT	0.699	0.000
	hs-cTnT	−0.501	0.017
	c-Isoprostanes	−0.516	0.014
KYNA	EF	−0.501	0.029
	SV	−0.558	0.016
	DT	−0.523	0.026
	hs-cTnT	0.641	0.004
3-HAA	EF	−0.611	0.003
	SV	−0.598	0.004
	DT	−0.470	0.031
	hs-cTnT	0.581	0.006
	c-Isoprostanes	0.523	0.015
Pigs			
TRP	CA1 lesions	−0.728	0.017
KYNA	MAP	−0.799	0.000
	CO	−0.742	0.000
3-HAA	MAP	−0.561	0.016
	CO	−0.729	0.001
	CA1 necrosis	0.792	0.006
	Cortical astrogliosis	0.778	0.008
	NDS	0.744	0.014

KP, kynurenine pathway; TRP, tryptophan; KYNA, kynurenic acid; 3-HAA, 3-hydroxyanthranilic acid; EF, ejection fraction; CO, cardiac output; SV, stroke volume; DT, deceleration time; hs-cTnT, high sensitive cardiac troponin T; c-Isoprostanes, cardiac isoprostanes; MAP, mean arterial pressure; CA1, CA1 hippocampal area; NDS, neurological deficit score.

healthy ones ($p < 0.01$, Fig. 2). Plasma levels of the TRP's metabolite KYN and its derivatives, KYNA and 3-HAA, significantly increased following resuscitation ($p < 0.01$ vs. healthy rats, Fig. 2). Plasma levels of TRP tended to return to normal values 3 days after resuscitation (Fig. 2). However, higher plasma concentrations of KYN, KYNA, and 3-HAA persisted ($p < 0.01$ vs. healthy rats, Fig. 2).

Post-resuscitation left ventricle (LV) systolic and diastolic dysfunction occurred in each rat, as evidenced by hemodynamic and echocardiographic data reported in Table 1. More specifically, marked decreases in mean arterial pressure (MAP) and coronary perfusion pressure, LV ejection fraction, stroke volume, and deceleration time of early mitral inflow, were observed at 2 and 4 h post-resuscitation ($p < 0.01$ vs. healthy rats, Table 1). Changes in the echocardiographic parameters, evaluating the severity of post-resuscitation LV dysfunction, were significantly and directly related to changes in TRP and significantly inversely related to changes in KYNA and 3-HAA (Table 2).

The severity of post-resuscitation myocardial injury was also reflected by the plasma levels of hs-cTnT and cardiac isoprostanes, that significantly increased at 2 and 4 h post-resuscitation ($p < 0.01$ vs. healthy rats, Table 1). Changes in plasma levels of these biomarkers of myocardial damage and oxidative stress were significantly inversely related to the concurrent changes in TRP and significantly directly related to changes in KYNA and 3-HAA (Table 2).

3.2. Pigs

Similarly to rats, plasma levels of TRP tended to decrease, while those of KYN tended to increase at 1 h and 5 days post-resuscitation (p not significant, Fig. 3). Plasma levels of KYNA and 3-HAA significantly increased at 1 h post-resuscitation compared to baseline values ($p < 0.01$, Fig. 3). These increased plasma concentrations of TRP's metabolites persisted 5 days later ($p < 0.05$, Fig. 3).

Post-resuscitation myocardial dysfunction developed in each pig, as evidenced by the hemodynamic measurements in Table 2. More specifically, lower MAP and LV cardiac output (CO) were observed at 1 and 4 h post-resuscitation ($p < 0.01$ vs. baseline, Table 1). These decreases in MAP and CO were significantly inversely related to the increases in KYNA and 3-HAA (Table 2). Neurological deficit score at day 1, 3 and 5 post-resuscitation is reported in Table 2, together with cerebral histopathological scores, i.e. cortical and CA1 hippocampal lesion, necrosis and astrogliosis. Early changes in 3-HAA were significantly directly related to the severity of neurological deficit score (supplemental Fig.) and to the histological alterations (Table 2). Overall changes in TRP were significantly inversely related to the severity of hippocampal lesions (Table 2).

3.3. Humans

Similarly to rats and pigs, there was a trend toward lower plasma levels of TRP and higher levels of KYN, in resuscitated patients in comparison to healthy volunteers (p not significant, Fig. 4). Plasma levels of KYNA and 3-HAA were significantly higher at 1 h post-resuscitation, compared to plasma concentrations in healthy volunteers ($p < 0.01$, Fig. 4), and tended to decrease during the following 3 days (Fig. 4). However, higher plasma levels of 3-HAA persisted 3 days later ($p < 0.05$ vs. healthy volunteers, Fig. 4). Only 2 of the 5 resuscitated patients survived till ICU discharge and presented lower plasma levels of KYNA and 3-HAA in comparison to those who died. Interestingly, plasma levels of 3-HAA were approximately double in patients who died compared to those who survived (190 ± 12 nM vs. 102 ± 12 nM).

Post-resuscitation circulating levels of proinflammatory cytokines, procalcitonin, and CRP are reported in the supplemental Table.

4. Discussion

This study demonstrates that kynurenine pathway is activated following resuscitation from cardiac arrest in three different species. Accordingly, KP activation was initially observed in resuscitated rats, was then validated in pigs subjected to cardiac arrest and CPR, and was ultimately confirmed in a small cohort of human patients. Indeed, increases in plasma levels of KP metabolites, KYN, KYNA and 3-HAA, occurred during the initial hours following resuscitation and persisted up to 3–5 days following cardiac arrest. KP activation showed an equivalent time course in rats, pigs, and humans, and was significantly related to the severity of post-resuscitation myocardial dysfunction, cerebral injury, functional outcome and survival.

KP is a major pathway of the catabolism of the essential aminoacid TRP. Specifically, two enzymes initiate the KP: tryptophan 2,3-dioxygenase (TDO), that is mainly present in the liver and is stimulated by glucocorticoids; and indoleamine 2,3-dioxygenase (IDO), that is widely expressed in a variety of human tissues, such as the brain, kidney, lung, spleen, and duodenum, as well as in macrophages and dendritic cells and is stimulated by proinflammatory cytokines, including interferon-γ, TNFa, IL-1 and 2, and by lipopolysaccharides and free radicals.[13,15] Indeed, upon inflammatory stimulation, IDO is induced and consequently KP activated.[13,14] Similarly to a sepsis-like syndrome, a systemic inflammatory and immune response is observed after CPR, and might be the trigger for IDO induction.[3,9,10,18] In the present study, early KP activation has been consistently observed in both small and large animals resuscitated from cardiac arrest, and ultimately confirmed in humans. In our study, systemic inflammation was present in the 5 resuscitated patients, as demonstrated by circulating levels of TNFa, IL-8,

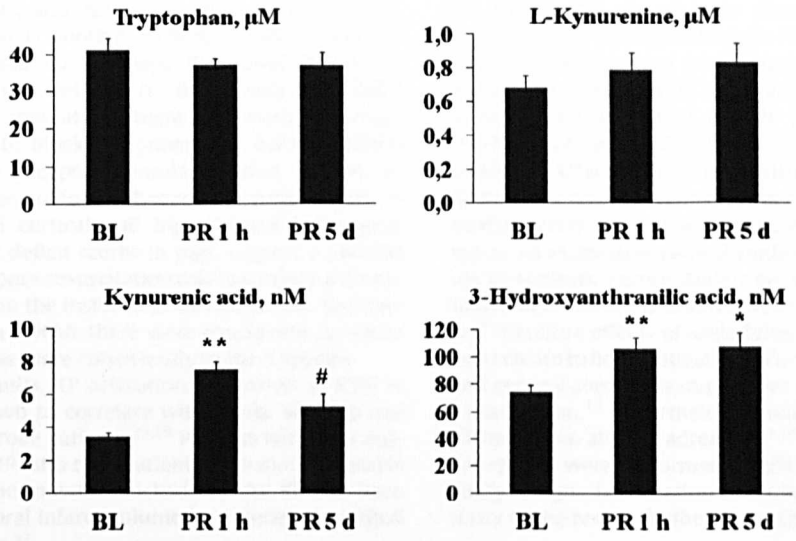

Fig. 3. Kynurenine pathway activation in pigs resuscitated from cardiac arrest. Plasma concentrations of trypthophan, L-kynurenine, kynurenic acid and 3-hydroxyanthranilic acid at baseline (BL) and after 1 h (h), and 5 days (d) post-resuscitation (PR). Data are reported as mean ± SEM. $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. BL; $^{\#}p < 0.05$ vs. PR 1 h.

MIF, and CRP, while association between KP activation and myocardial oxydative stress, i.e. isoprostanes, has been observed in the rat model.

Altered TRP metabolism has been described as a hallmark of many stress related situations.[15,22] In these settings of glucocorticoid overdrive, in fact, TDO is activated together with a super induction of IDO in response to stress.[15] Concurrently elevated circulating catecholamines induce an enhanced uptake of free TRP contributing to reduction in total TRP concentrations.[15] Thus, rodents subjected to stressful conditions, i.e. forced swimming or immobilization, a low TRP diet increased adrenal weight, plasma corticosterone levels and reactivity to stimuli; administration of TRP, instead, had acute antianxiety-like effects.[15,23] Indeed, cardiac arrest is characterized by increases in plasma cortisol level and catecholamine release. A trend toward lower serum cortisol level in survivors than in non survivors has been earlier reported.[15,24] The stress of whole body ischemia/reperfusion that follows cardiac arrest might be therefore another component affecting the described KP activation after CPR.

It has been proposed that TRP catabolism through the KP may contribute to oxidative stress and brain damage following ischemia.[25] Contributors to the development of ischemia-induced cerebral injury are, in fact, post-ischemia neuroimmune and inflammatory reactions.[3,26] Accordingly, one component of this network is the KP.[25,27] Indeed, KYN is the first KP metabolite, that is further metabolized to the neurotoxic 3-HAA and its derivatives, quinolinic acid and picolinic acid (QA and PA). 3-HAA exerts its neurotoxic actions by inducing both cerebral oxidative stress and excitotoxicity through activation of N-Methyl-D-Aspartate (NMDA)-receptors by QA and PA. 3-HAA's metabolites have not only neuroexcitatory effects, but also neurotoxic ones, causing neuronal, astrocyte and microglial cell injury, destruction

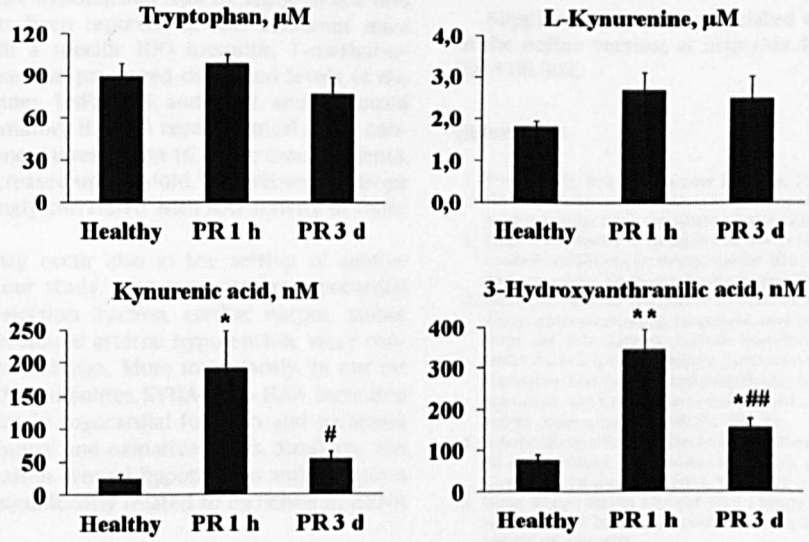

Fig. 4. Kynurenine pathway activation in men resuscitated from cardiac arrest. Plasma concentrations of trypthophan, L-kynurenine, kynurenic acid and 3-hydroxyanthranilic acid in healthy volunteers and in men after 1 h (h), and 3 days (d) post-resuscitation (PR). Data are reported as mean ± SEM. $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. BL; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ vs. PR 1 h.

of postsynaptic elements, and reductions in cerebral cholinergic circuits.[25,27–29] KYNA is another KYN metabolite, produced by astrocytes, in response to increased KYN level. KYNA has neuroprotective properties related to its activity as NMDA antagonist.[29–31] KYNA generation might represent an adaptive response in order to block the potentially harmful effects of excessive glutamate receptor stimulation that follows an ischemic insult.[26] The correlations between post-resuscitation 3-HAA plasma levels and cortical and hippocampal histological lesions and neurological deficit scores in pigs, suggest a possible role of KP metabolites in post-resuscitation cerebral injury and neurogical dysfunction also in the instance of cardiac arrest. Together with increases in plasma 3-HAA, there were concurrent increases in plasma KYNA and these were consistently in the 3 species.

In support of our results, KP activation, expressed as KYN to TRP ratio, has been shown to correlate with stroke severity and long-term outcome in stroke patients.[25,28] Patients with poor outcome had higher KYN/TRP ratio than patients with more favorable outcome. Moreover, experimental blockade of the KP has been reported to reduce cerebral infarct volume in a model of cerebral ischemia and reperfusion.[32]

More importantly, KP activation has been associated not only with brain injury but it may also concur to the pathogenesis of systemic inflammatory response syndrome and sepsis.[14,16,29,33,34] IDO activity was markedly increased in 132 patients with bacteremia, in whom KP was significantly more activated in non-survivors compared to survivors.[16] In 60 major trauma patients, significantly increased KYN was detectable already within 24 h after hospital admission in blood from patients who later developed sepsis.[35] In those patients KP activation predicted subsequent sepsis development, multiple organ failure, and survival. Most likely early post-traumatic inflammation in conjunction with augmented circulating pro-inflammatory cytokines and neutrophil activation resulted in IDO stimulation and consequent KP activation. The above events may occur also in cardiac arrest patients.[3,9,10]

KP also concurs to the pathogenesis of vasoplegia and hypotension during septic shock.[14,33,34] IDO expression was, in fact, induced in the endothelial cells of small resistance vessels and contributed to the dysregulation of vascular tone during systemic inflammation due to endotoxemia in mice.[33] This increased IDO activity accounted for a greater production of KYN, that directly mediated arterial relaxation through soluble guanylate cyclase activation.[14] A clear protection against hypotension due to septic shock and a reduced mortality has been reported in IDO knockout mice or in mice treated with a specific IDO inhibitor, 1-methyl-D-tryptophan.[34] Those mice also presented decreased levels of the pro-inflammatory cytokines, TNFa, IL-6, and IL-12, and enhanced levels of the anti-inflammatory IL-10. A recent clinical study confirmed the above experimental results on 16 septic shock patients, in which IDO activity increased up to 9-fold.[14] Moreover, inotrope requirements were strongly correlated with IDO activity in those septic patients.[14]

The above events may occur also in the setting of cardiac arrest.[3,10] As shown in our study, post-resuscitation myocardial dysfunction, with low ejection fraction, cardiac output, stroke volume, together with profound arterial hypotension, were consistently reported in rats and pigs. More importantly, in our rat model, plasma levels of KP metabolites, KYNA and 3-HAA, increased concurrently to decreases in myocardial function and increases in biomarkers of heart injury and oxidative stress. Similarly, the severity of post-resuscitation arterial hypotension and reduction in cardiac output were significantly related to increases in KYNA and 3-HAA in pigs.

We recognize limitations in the interpretations of our findings. First, the experimental design was purely observational and focused on demonstration of post cardiac arrest activation of KP, thus it did not allow to identify causal relations: changes in circulating molecules may be the consequence as well as the cause of the observed pathophysiologic alterations. In order to investigate direct effects of KP activation on outcome of cardiac arrest, experiments that include administration of specific IDO inhibitors are planned. Nevertheless, this is the first evidence of consistent KP activation after cardiac arrest in animals and humans. Second, the results obtained from the 5 patients resuscitated from cardiac arrest have to be considered as a "proof of concept" and not as an exahustive clinical confirmation, due to the small number of subjects. Future studies on a larger number of patients are underway. Third, the studies were performed in healthy animals and therefore effects of underlying coronary disease in KP activation remain to be investigated.[36] Fourth, animals were anesthetized and general anesthesia might have influenced stress response and KP activation.[13] Nevertheless, implications of the above factors on KP have been already adressed.[13,36] Last, the studies on the different species were performed at different Institutions with different study designs, i.e. duration of cardiac arrest. However, the consistency of the results further strengthens our findings.

5. Conclusions

This study demonstrates that KP is activated early in the post-resuscitation and persists during the initial 3–5 days post-cardiac arrest, in rats, pigs, and humans. KP activation is significantly related to the severity of post-resuscitation myocardial dysfunction, cerebral injury, functional outcome and survival.

Conflict of interest statement

Authors have no conflict of interest.

Acknowledgments

The first three authors, GR, MF, and LB, played equal roles.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.resuscitation.2013.06.002.

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Clinical paper

Amplitude spectrum area to guide resuscitation—A retrospective analysis during out-of-hospital cardiopulmonary resuscitation in 609 patients with ventricular fibrillation cardiac arrest☆

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ABSTRACT

Introduction: The capability of amplitude spectrum area (AMSA) to predict the success of defibrillation (DF) was retrospectively evaluated in a large database of out-of-hospital cardiac arrests.
Methods: Electrocardiographic data, including 1260 DFs, were obtained from 609 cardiac arrest patients due to ventricular fibrillation. AMSA sensitivity, specificity, accuracy, and positive and negative predictive values (PPV, NPV) for predicting DF success were calculated, together with receiver operating characteristic (ROC) curves. Successful DF was defined as the presence of spontaneous rhythm ≥40 bpm starting within 60 s from the DF. In 303 patients with chest compression (CC) depth data collected with an accelerometer, changes in AMSA were analyzed in relationship to CC depth.
Results: AMSA was significantly higher prior to a successful DF than prior to an unsuccessful DF (15.6 ± 0.6 vs. 7.97 ± 0.2 mV-Hz, $p < 0.0001$). Intersection of sensitivity, specificity and accuracy curves identified a threshold AMSA of 10 mV-Hz to predict DF success with a balanced sensitivity, specificity and accuracy of almost 80%. Higher AMSA thresholds were associated with further increases in accuracy, specificity and PPV. AMSA of 17 mV-Hz predicted DF success in two third of instances (PPV of 67%). Low AMSA, instead, predicted unsuccessful DFs with high sensitivity and NPV >97%. Area under the ROC curve was 0.84. CC depth affected AMSA value. When depth was <1.75 in., AMSA decreased for consecutive DFs, while it increased when the depth was >1.75 in. ($p < 0.05$).
Conclusions: AMSA could be a useful tool to guide CPR interventions and predict the optimal timing of DF.

1. Introduction

Cardiopulmonary resuscitation (CPR) in conjunction with electrical defibrillation (DF) can re-establish spontaneous circulation (ROSC) after cardiac arrest from ventricular fibrillation (VF) and pulseless ventricular tachycardia (VT).[1,2] However, despite major efforts to improve outcomes from cardiac arrest, survival rates remains dismal.[3–5] Major factors contributing to poor outcomes include delays in CPR, ineffective and frequently interrupted chest compressions (CC), and limited access to, or delayed DF.[2,6,7]

Timing of DF in relationship to CC has been a subject of major interest. Based on available evidence, the 2005 guidelines recommended an initial interval of CC prior to DF, especially when the duration of untreated cardiac arrest exceeded 4 mins.[1,8] Nevertheless, the recent 2010 guidelines highlighted the insufficient evidence to support or refute CPR before DF and called again for early DF.[9–11] Subsequent DF has been recommended to be attempted on a time based protocol, i.e. after every 2 min cycle of CC,[9] which may lead to futile DF attempts and unnecessary CC interruptions, potentially creating worse outcome.[12–16] The timing of DF is even more difficult in the instance of recurrence of VF.[17]

The onset time of VF is rarely known, especially in the out-of-hospital setting, making it difficult to determine the priority of CPR intervention based on the duration of the untreated cardiac arrest. There is also insufficient knowledge about the optimal duration of the CC interval prior to DF. The decision whether to interrupt

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CC to deliver a DF is therefore difficult. Electrocardiographic (ECG) analysis of the VF waveform might represent the best non-invasive decision guide.

The "Amplitude Spectrum Area" (AMSA) has been demonstrated to be one of the most accurate predictors for successful DF, in both animal and small retrospective clinical studies.[18–22] In the present study, we retrospectively evaluated the ability of AMSA to predict DF success in a large database of out-of-hospital VFs. We hypothesized that AMSA, derived from conventional AED pads, would be an useful indicator to predict DF success and guide CPR interventions. We further hypothesized that AMSA could serve as a monitor of CC quality.

2. Methods

A database of ECG traces recorded during pre-hospital CPR, including 1410 DFs, obtained from 748 patients between 2005 and 2007, was available through the courtesy of ZOLL Medical Corporation (Chelmsford, MA, USA). ECGs were recorded from defibrillation pads using ZOLL AED PLUS and ZOLL AED PRO in multiple emergency medical systems in the United States through a regular field case submission program. The electronic data did not contain any patient identifiable information, accordingly to Health Insurance Portability and Accountability Act (HIPAA) regulations.

ECGs were recorded at a sample rate of 250 Hz. The AEDs provided a single rectilinear biphasic waveform shock of 120 J for the first DF, and 150 or 200 J for the subsequent DFs. The AMSA analysis has been previously described.[18–21,23] Briefly, ECG signals were processed using a 2 Hz high-pass filter to minimize low frequency artifacts produced by CC and a 48 Hz low-pass filter to remove interference of ambient noise at higher frequencies. Analog ECG signals were digitized and converted from a time to a frequency domain by fast Fourier transformation. AMSA was calculated as the sum of the products of individual frequencies and their amplitudes (Supplemental Fig. 1), i.e. $AMSA = \sum A_i F_i$, where A_i represented the amplitude at ith frequency F_i.

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.resuscitation.2013.08.017.

The analysis was performed during hands off time on a 512 point window (2.05 s) ending 0.5 s prior to the DF. A Tukey FFT window was used to reduce edge effects. For the purpose of this study, the DF outcome was defined according to the following established criteria:[21] "successful defibrillation" or return of a potentially perfusing rhythm, if DF restored an organized rhythm with heart rate ≥ 40 beats/min commencing within 60 s post shock; and "unsuccessful defibrillation" or failure of return of a potentially perfusing rhythm, if VF/VT, asystole, or pulseless electrical activity with pauses > 5 s occurred. Only ECG recordings with adequate pre- and post-DF durations and in which DF outcome could be confirmed were included in the study: 1260 instances from 609 patients. In addition, subsequent DFs were classified as being for recurrent VF if the preceding DF was successful, or for refractory VF if the preceding DF had failed to restore a potentially perfusing rhythm.[17] A sub-group analysis was conducted on DFs obtained from 303 patients, for whom CC depth data were available. Depth of CC was measured from an accelerometer used for CPR feedback and registered by the AEDs. Changes in AMSA between consecutive DFs were analyzed in relationship to the 2005 recommended CC depth > 1.5 in.[8]

2.1. Statistical analysis

AMSA was computed using Matlab 7.2 (MathWorks, Natick, MA). Two independent readers reviewed the ECG recordings to confirm DF outcomes. Differences in AMSA between DFs were analyzed by analysis of variance (ANOVA) with Scheffe's method for multiple comparisons. A range of AMSA thresholds was evaluated. The sensitivity, defined as the capability of AMSA to identify DFs that successfully reestablished a potentially perfusing rhythm, was calculated as the number of correctly predicted successful DFs divided by the total number of successful DFs. The specificity, which refers to the capability of AMSA to identify failure of a DF, was calculated as the number of correctly predicted unsuccessful DFs divided by total number of unsuccessful DFs. The positive predictive value (PPV) referred to the proportion of DFs that were correctly predicted by AMSA to restore a potentially perfusing rhythm. The negative predictive value (NPV) represented the proportion of DFs that were correctly predicted by AMSA to fail. The accuracy was calculated as the proportion of true results (both true positively predicted successful DFs and true negatively predicted unsuccessful DFs) in the population. Finally, receiver operator characteristic (ROC) curve analysis was performed. SPSS 16.0 (SPSS Inc., Chicago, IL) was used. A value of $p < 0.05$ was regarded as statistically significant. Data are presented as mean ± SEM.

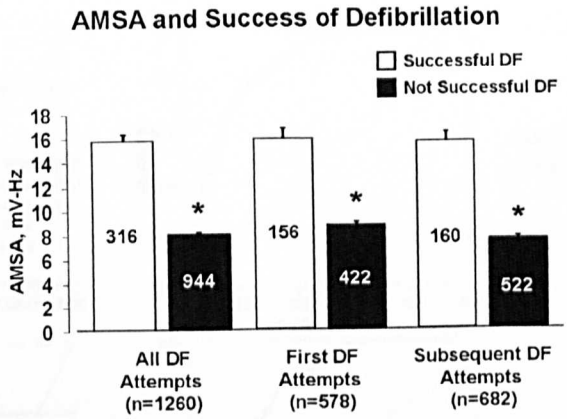

Fig. 1. AMSA values for successful and not successful DF for all, first and subsequent attempts. Number of attempts are reported inside bars. *$p < 0.0001$ between successful and not successful DFs.

3. Results

A total of 1260 DFs, including 578 first attempts and 682 subsequent ones from 609 patients, were included in the analyses.

3.1. All DF attempts

Among all 1260 DFs, 316 were successful (25.1%), while 944 were unsuccessful (74.9%). AMSA was significantly higher prior to a successful DF than prior to an unsuccessful one (15.6 ± 0.6 vs. 7.97 ± 0.2 mV-Hz, $p < 0.0001$, Fig. 1).

Using the intersection of sensitivity, specificity, and accuracy curves (Fig. 2A), an AMSA threshold of 9.8 mV-Hz, provided a balanced sensitivity, specificity and accuracy of 78%, with a NPV of 91% and a PPV of 54%. An AMSA threshold of 14 mV-Hz provided the highest accuracy (80%) in predicting DF outcome, with a PPV of 61% and a specificity of 90%. Higher AMSA thresholds were associated with further increases in PPV and specificity. An AMSA threshold of 17 mV-Hz resulted in the highest PPV (67%) in predicting DF success.

For low AMSA thresholds, the majority of unsuccessful DFs were correctly predicted with high sensitivity and NPV (Fig. 2A). Using AMSA < 7 mV-Hz as cutoff, more than 42% of unsuccessful DFs might have been avoided with a NPV > 97% (Fig. 2A and Supplemental Fig. 2). Lower thresholds further improved NPV.

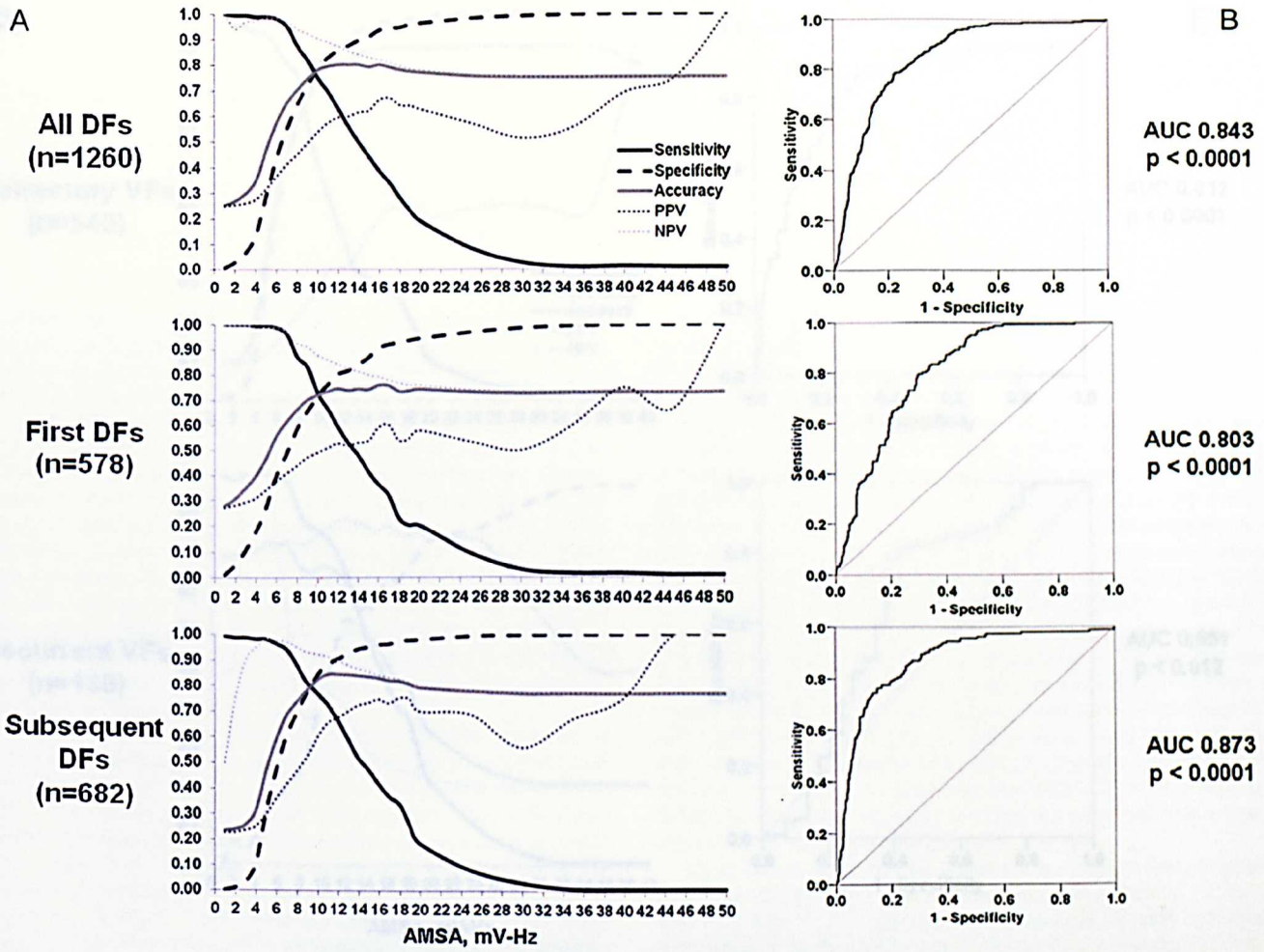

Fig. 2. (A) Sensitivity, specificity, accuracy, and positive and negative predictive value (PPV and NPV) curves for different AMSA values, in all DFs, first DFs, and subsequent DFs. (B) ROC curves for AMSA and DF outcome prediction for all, first, and subsequent DFs.

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Area under the ROC curve was 0.84 ($p < 0.0001$, Fig. 2B).

3.2. First DF attempts

Among 578 first DFs, 156 were successful (27%), while 422 were unsuccessful (73%). AMSA was significantly higher prior to a successful DF than prior to an unsuccessful one (15.7 ± 0.9 mV-Hz vs. 8.7 ± 0.3, $p < 0.0001$, Fig. 1).

Using the intersection of sensitivity, specificity, and accuracy curves (Fig. 2A), an AMSA threshold of 10.2 mV-Hz provided a balanced sensitivity, specificity and accuracy of 73%, with a NPV of 88% and a PPV of 50%. An AMSA threshold of 17 mV-Hz resulted in the highest accuracy (76%) and PPV (60%) in predicting DF success, with a specificity of 92%.

With an AMSA < 7 mV-Hz, more than 40% of unsuccessful DFs might have been avoided with a NPV > 97% (Fig. 2A and Supplemental Fig. 2). Lower thresholds further improved NPV.

Area under the ROC curve was 0.80 ($p < 0.0001$, Fig. 2B).

3.3. Subsequent DF attempts

Among 682 subsequent DFs, 160 were successful (23.5%), while 522 were unsuccessful (76.5%). AMSA was significantly higher prior to a successful DF than prior to an unsuccessful one (15.5 ± 0.8 mV-Hz, $p < 0.0001$, Fig. 1).

Using the intersection of sensitivity, specificity, and accuracy curves (Fig. 2A), an AMSA threshold of 9.2 mV-Hz provided a balanced sensitivity, specificity and accuracy of 79%, with a NPV of 93% and a PPV of 54%. An AMSA threshold of 14 mV-Hz resulted in the highest accuracy (84%) in predicting DF success, with a PPV of 72% and a specificity of 94%. Further increases in AMSA threshold were associated with further increases in PPV and specificity. An AMSA threshold of 18 mV-Hz resulted in the highest PPV (75%) in predicting DF success.

With an AMSA < 7 mV-Hz, more than 46% of unsuccessful DFs might have been avoided with a NPV > 97% (Fig. 2A and Supplemental Fig. 2). Lower thresholds further improved NPV.

Area under the ROC curve was 0.87 ($p < 0.0001$, Fig. 2B).

3.4. DF attempts for recurrent and refractory VF

Among 682 subsequent DF attempts, 139 were delivered for recurrent VF, while 543 were for refractory VFs. Among 139 DFs delivered for recurrent VF, 110 were successful (79.1%), while of 543 DFs delivered for refractory VF, only 50 were successful (9.2%) (Table 1). AMSA was significantly higher in recurrent VF than in refractory VF (16.2 ± 0.9 vs. 7.6 ± 0.2 mV-Hz, $p < 0.0001$, Table 1). In the instance of recurrent VF, there was no significant difference in

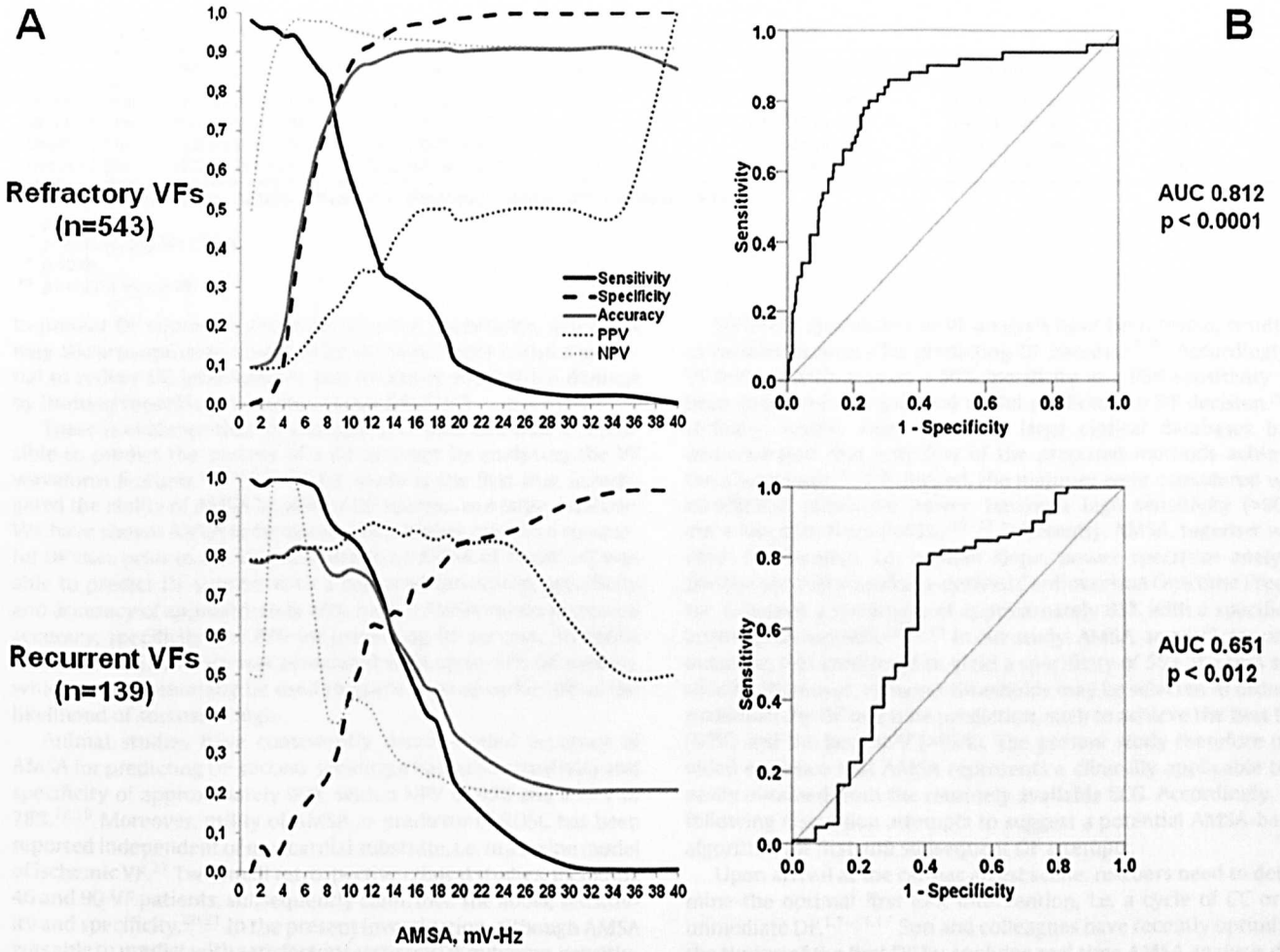

Fig. 3. (A) Sensitivity, specificity, accuracy, and positive and negative predictive value (PPV and NPV) curves for different AMSA values, in DFs delivered for refractory and recurrent VFs. (B) ROC curve for AMSA and DF outcome prediction for DFs delivered for refractory and recurrent VFs.

AMSA prior to a successful DF compared to that prior to an unsuccessful one (16.8 vs. 13.8 mV-Hz, Table 1). For refractory VFs, AMSA was significantly higher prior to a successful DF than prior to an unsuccessful one (12.7 ± 1 vs. 7 ± 0.2 mV-Hz, $p < 0.0001$, Table 1). AMSA was accurate for predicting DF outcome only in the instance of refractory VF (Fig. 3).

In the instance of refractory VF, the intersection of sensitivity, specificity, and accuracy curves provided an AMSA threshold of 8.5 mV-Hz accounting for a balanced sensitivity, specificity and accuracy of 77% (Fig. 3A). An AMSA threshold of 17 mV-Hz resulted in peak accuracy (91%) and peak PPV (50%), with a specificity of 97%.

Table 1
AMSA prior defibrillation for refractory and recurrent VF.

	Refractory VF ($n = 543$)	Recurrent VF ($n = 139$)
Mean AMSA, mV-Hz	7.6 ± 0.2	16.2 ± 0.9*
AMSA prior to successful DFs, mV-Hz	12.7 ± 1	16.8 ± 1
AMSA prior to failing DFs, mV-Hz	7.0 ± 0.2#	13.8 ± 1.8
Successful DFs, % (n)	9.2 (50/543)	79.1 (110/139)

DFs, defibrillation attempts; VF, ventricular fibrillation; and mean ± SEM.
* $p < 0.0001$ vs. refractory VF.
# $p < 0.0001$ vs. successful DFs.

Area under the ROC curve was 0.81 ($p < 0.0001$) for refractory VF, and 0.65 for recurrent VF ($p = 0.012$, Fig. 3B).

3.5. Influence of CC depth and interruptions on AMSA

When CC depth was <1.75 in., AMSA decreased by 3.9 mV-Hz between the first and the second DF and further decreased between the second and the third attempt (Table 2, $p < 0.001$). By contrast, when CC depth was >1.75 in., AMSA was equivalent for the first and the second DF ($p = 0.47$) and then increased by 1.7 mV-Hz between the second and the third attempt. A similar but less pronounced trend was observed with a CC depth cutoff at 1.5 in. (Table 2).

AMSA significantly decreased during the 16 s pre-DF CC interruption for AED rhythm analyses and capacitor charge ($p < 0.01$, Fig. 4). AMSA decreased even more during CC interruptions prior to first DF ($p < 0.01$). In subsequent DFs, AMSA decrease during CC interruptions was less pronounced ($p < 0.05$ only after 12 s CC interruption).

4. Discussion

Current DF algorithms are static in the sense that they do not consider the passage of time and the pathophysiology of the arrested myocardium.[9,24] In our data, the standard DF approach led to a successful DF in only 25% of attempts. The ability of AMSA

Table 2
AMSA value changes among the initial 3 defibrillation attempts in relationship to chest compression depth.

	AMSA 1st DF (mV-Hz)	AMSA 2nd DF (mV-Hz)	ΔAMSA 2nd–1st DF	AMSA 2nd DF (mV-Hz)	AMSA 3rd DF (mV-Hz)	ΔAMSA 3rd–2nd DF
Depth > 1.75 in.	11.2 ± 0.8 (*n* = 155)	10.4 ± 0.8 (*n* = 155)	−0.7	8.1 ± 0.5 (*n* = 81)	9.7 ± 0.8 (*n* = 81)	+1.7
Depth < 1.75 in.	13.3 ± 0.9 (*n* = 148)	9.4 ± 0.4 (*n* = 148)##	−3.9*	8.8 ± 0.5 (*n* = 80)	8.4 ± 0.5 (*n* = 80)	−0.4**
Depth > 1.5 in.	11.9 ± 0.8 (*n* = 192)	10.1 ± 0.7 (*n* = 192)	−1.6	8.1 ± 0.4 (*n* = 96)	9.5 ± 0.7 (*n* = 96)	+1.5
Depth < 1.5 in.	12.8 ± 1 (*n* = 111)	9.5 ± 0.5 (*n* = 111)#	−3.3	9 ± 0.6 (*n* = 65)	8.5 ± 0.6 (*n* = 65)	−0.6**

DFs, defibrillation attempts; ΔAMSA, difference in AMSA value between DFs; and mean ± SEM.
* $p < 0.02$.
** $p < 0.01$ vs. depth > 1.75 in.
\# $p < 0.01$.
\#\# $p < 0.0001$ vs. 1st DF.

to predict DF success is therefore of great importance, since this may allow to optimize timing of DF delivery. There is also a potential to reduce CC interruptions and minimize myocardial damage by limiting repetitive and unnecessary DFs.[12–16]

There is evidence that VF changes over time and that it is possible to predict the success of a DF attempt by analysing the VF waveform features.[9,17–23,25,26] Our study is the first that investigated the ability of AMSA to predict DF success on a large database. We have shown AMSA to be significantly higher prior to a successful DF than prior to a failing one. While an AMSA of 10 mV-Hz was able to predict DF success with a balanced sensitivity, specificity and accuracy of approximately 80%, higher AMSA values increased accuracy, specificity and PPV for predicting DF success. An AMSA threshold of 17 mV-Hz was associated with up to 67% DF success, which could potentially be used to guide toward earlier DF as the likelihood of success is high.

Animal studies have consistently demonstrated accuracy of AMSA for predicting DF success, yielding a balanced sensitivity and specificity of approximately 90%, with a NPV of 95% and a PPV of 78%.[18,19] Moreover, utility of AMSA as predictor of ROSC has been reported independent of myocardial substrate, i.e. in a swine model of ischemic VF.[27] Two small retrospective clinical studies, including 46 and 90 VF patients, subsequently confirmed the above sensitivity and specificity.[20,21] In the present investigation, although AMSA was able to predict with satisfactory accuracy DF outcome, sensitivity, specificity, and PPV were lower compared to the earlier reports. We believe, however, that current results are more realistic due to the large database employed, that is approximately 10-fold greater than earlier ones and represents one of the largest studied to now for VF waveform analysis. Indeed, a good DF predictor should be both sensible and specific. For this reason, a balanced sensitivity and specificity of 80%, as observed in our results, makes AMSA a useful guide for DF decision.

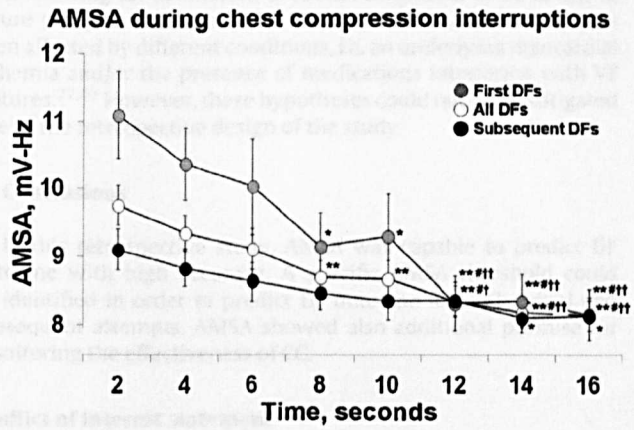

Fig. 4. AMSA changes measured every 2 s during the 16 s pre-DF "hands off" period for all, first and subsequent DFs. *$p < 0.05$ and ** $p < 0.01$ vs. 2 s; #$p < 0.01$ vs. 4 s; †$p < 0.05$ and ††$p < 0.01$ vs. 6 s.

Different approaches to VF analysis have been tested, resulting in variable accuracy for predicting DF success.[28–35] Accordingly, a VF feature with at least a 50% specificity at a 95% sensitivity has been suggested as a safe and useful predictor for DF decision.[29,30] Unlikely, results from relatively large clinical databases have demonstrated that only few of the proposed methods achieved the above limit.[29,34,35] Indeed, the majority were considered with insufficient predictive power, having a high sensitivity (>90%), but a low specificity (<40%).[30–33] Differently, AMSA, together with other VF features, i.e. median slope, power spectrum analysis, and the wavelet transform-derived Cardioversion Outcome Predictor, achieved a sensitivity of approximately 95% with a specificity between 56 and 66%.[29,34,35] In our study, AMSA, as predictor of DF outcome, was confirmed to yield a specificity of 55% at a 95% sensitivity. Moreover, different thresholds may be selected in order to maximize the DF outcome prediction, such to achieve the best PPV (67%) and the best NPV (>95%). The present study therefore provided evidence that AMSA represents a clinically applicable tool, easily obtained from the routinely available ECG. Accordingly, the following discussion attempts to suggest a potential AMSA-based algorithm for first and subsequent DF attempts.

Upon arrival at the cardiac arrest scene, rescuers need to determine the optimal first CPR intervention, i.e. a cycle of CC or an immediate DF.[1,7–11,17] Sun and colleagues have recently optimized the timing of the first DF by applying real time AMSA analysis during CC in a rat model of VF.[26] DF was attempted only when AMSA achieved a predefined threshold predictive of successful shock. With that approach, 70% of animals were resuscitated after the first DF, in contrast to 0% when DF was attempted following standard guidelines. AMSA-guided CPR also significantly reduced CC interruptions and ultimately improved post resuscitation myocardial and neurologic function and survival. In our population, interrupting CC for delivery of DF at an AMSA of 17 mV-Hz could have raised first DF success up to 60% compared to 27% observed with the standard approach.

AMSA also has predictive ability for subsequent DF attempts, but is dependent upon the type of VF. Subsequent DFs may be due to either VF refractory to earlier DFs or to a recurrence of VF after an initially successful DF. Similar to an earlier small retrospective study on 44 patients,[17] our study demonstrated that AMSA was accurate for DF prediction only in the instance of refractory VF. The AMSA value to be considered as a threshold for delivery of a DF with the highest likelihood of success was again 17 mV-Hz. AMSA therefore might be an useful decision tool for the treatment of refractory VF. Recurrent VF, on the other hand, presented an overall high AMSA, with no differences between successful and unsuccessful DFs, and an elevated DF success of almost 80%. Recurrent VF will probably not benefit from an initial interval of CC, but rather should receive DF immediately, similar to a witnessed arrest.

The total electrical energy delivered with repetitive DFs might be an important determinant for the severity of post resuscitation myocardial dysfunction and survival.[12,36] Using AMSA lower than 7 mV-Hz as guidance, more than 45% of unsuccessful and potentially detrimental DFs might have been avoided. This approach

could also reduce unnecessary CC interruptions for the delivery of futile DFs. Even minimal interruptions of less than 4 s can generate declines in myocardial perfusion to near 40%.[37] In our population, pre-DF pauses caused significant decreases in AMSA, likely related to the reduced myocardial perfusion and ultimately anticipating decreases in DF success.[22,23] Limiting the frequency and duration of such CC interruptions may improve clinically relevant outcomes in cardiac arrest.[9,13,15,16]

AMSA appears to carry the additional promise of being able to monitor the effectiveness of CC. The present analyses have demonstrated that AMSA decreased between consecutive shocks during shallow CC, while it increased when CC was of greater depth. We have previously reported the possibility of assessing CC depth utilizing AMSA in a porcine model, in which animals were randomized to optimal or suboptimal depth of CC.[23] Similarly to CPP, AMSA thresholds achieved were contingent on the depth of compressions such that AMSA increased progressively during CC, and predicted the likelihood of successful DF.[23] Therefore, when future technology allows for real time AMSA analyses during CC, a rescuer may be prompted to push harder if the AMSA value is too low.[38]

Based on our results the following DF decision, based on a continuous real-time AMSA analyses during uninterrupted CC, could be beneficial. In the presence of a very low AMSA, i.e. below 7 mV-Hz, the likelihood to deliver a successful DF is low. CC therefore should be performed until AMSA is deemed to be favorable for a DF, minimizing interruptions in CC and delivery of unnecessary shocks. The decision of interrupting CC to attempt a DF should be limited to when there is the highest probability of success. DF may be delivered as initial intervention or during CC even before completion of a regular 2 min cycle only when AMSA reaches the threshold of 17 mV-Hz. If AMSA values fall in the range between 7 and 17 mV-Hz, even though specificity and accuracy of shock success prediction are high, CPR should be performed accordingly to guidelines. Finally, recurrent VF should always receive an immediate DF.

We recognize important limitations. First, this was a retrospective analysis. Second, the study was performed on AED records and thereby data on duration of untreated VF, period of CC prior to first DF, patients' characteristics, co-morbidities, outcome and survival were not available. Nevertheless, earlier investigations have shown that peri-arrest factors, i.e. patients' age, sex and presenting rhythm, ambulance response time and presence of bystander CPR, did not significantly improve prediction accuracy.[39] Although, patients' age was not known in our database, since all the DF attempts had energy $\geq$ 120 J, all the patients might be considered as adult patients. Third, AMSA was calculated only during the pre-DF hands off time. Nevertheless, a large database was available, allowing for estimation of robust thresholds to be tested in future prospective evaluations. Finally, AMSA values might have been affected by different conditions, i.e. an underlying myocardial ischemia and/or the presence of medications interfering with VF features.[27,40] However, these hypotheses could not be investigated due to the retrospective design of the study.

5. Conclusions

In this retrospective study, AMSA was capable to predict DF outcome with high accuracy. A specific AMSA threshold could be identified in order to predict DF outcome for both initial and subsequent attempts. AMSA showed also additional promise for monitoring the effectiveness of CC.

Conflict of interest statement

Author W.Q. is employee of ZOLL Medical Corp. The other authors have no conflicts.

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The Best Timing For Defibrillation In Shockable Cardiac Arrest

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Abstract: High quality cardiopulmonary resuscitation (CPR, i.e. chest compressions and ventilations) and prompt defibrillation when appropriate (i.e. in ventricular fibrillation and pulseless ventricular tachycardia, VF/VT) are currently the best early treatment for cardiac arrest (CA). In cases of prolonged CA due to shockable rhythms, it is reasonable to presume that a period of CPR before defibrillation could partially revert the metabolic and hemodynamic deteriorations imposed to the heart by the no flow state, thus increasing the chances of successful defibrillation. Despite supporting early evidences in CA cases in which Emergency Medical System response time was longer than 5 minutes, recent studies have failed to confirm a survival benefit of routine CPR before defibrillation. These data have imposed a change in guidelines from 2005 to 2010. To take in account all the variables encountered when treating CA (heart condition before CA, time elapsed, metabolic and hemodynamic changes, efficacy of CPR, responsiveness to defibrillation attempt), it would be very helpful to have a real-time and non invasive tool able to predict the chances of defibrillation success. Recent evidences have suggested that ECG waveform analysis of VF, such as the derived Amplitude Spectrum Area, can fit the purpose of monitoring the CPR effectiveness and predicting the responsiveness to defibrillation. While awaiting clinical studies confirming this promising approach, CPR performed according to high quality standard and with minimal interruptions together with early defibrillation are the best immediate way to achieve resuscitation in CA due to shochable rhythms.

Keywords: cardiac arrest, cardiopulmonary resuscitation, ventricular fibrillation, defibrillation, waveform analysis

Main abbreviations: CPR, Cardiopulmonary Resuscitation; CA, cardiac arrest; VF, ventricular fibrillation; VT pulseless ventricular tachycardia; EMS, emergency medical system; ROSC, return to spontaneous circulation; StHD, survival to hospital discharge, AMSA, amplitude spectrum area.

Introduction

Treatment of cardiac arrest (CA) is a difficult challenge. Once CA has occurred, whether in- or out- of-hospital setting, chances of survival are still low despite efforts in research and implementation [1,2]. Options for effective treatment are substantially restricted to and conditioned by a few early interventions: immediate high quality cardiopulmonary resuscitation (CPR, i.e. chest compressions and ventilations) and, if appropriate, rapid defibrillation). Progress has been made in defining the importance of what makes the quality of CPR "good" enough to improve survival. On the other hand, the technical elements of "good defibrillation" have also been established [3]. However, it is possible that a better integration of these two interventions, with some grade of flexibility to suit each specific CA instance, might improve resuscitation success.

Initial rhythm is a major determinant of outcome since CA sustained by shockable rhythms, i.e. Ventricular Fibrillation and pulseless Ventricular Tachycardia (VF/VT), has a far better outcome in terms of survival to hospital discharge (StHD) when compared with not shockable rhytms, i.e. Asystole and Pulseless Electrical Activity (PEA) [4].

Therefore, even if a decline in the prevalence of VF/VT in out-of-hospital CA has been observed over the last 20 years [5,6], it seems important to optimize strategies to improve defibrillation effectiveness.

The aim of this article is to present the background of current guidelines together with recent studies about the best timing for defibrillation during CA; secondly, we will offer the reader an update about possible developments in the field.

"You are here": the background of current recommendations.

Best practice in resuscitation is regularly reviewed every five years by the International Liaison Committee on Resuscitation (ILCOR, a world-wide experts' network) [7] and translated into guidelines adapted to local needs (here we will refer to the European Resuscitation Council Guidelines for Resuscitation) [8]. For each topic of interest, a standardized systematic review is performed providing the basis for consensus on treatment.

A major effort in writing resuscitation guidelines has always been to keep them simple. "Time is life" when CA occurs, bystanders can widely vary in their role and experience and wasting minutes trying to follow complicated flow charts can be extremely deleterious. However exceptions can be admitted if the context can be easily delimited.

In the last two guidelines editions (2005 and 2010), a specific question focused on the possibility to improve defibrillation success in patients with prolonged CA found with a shockable rhythm. In these cases, the concern is that immediate defibrillation can find the heart in a state of poor "responsiveness" due to hypoxia, acidosis, metabolic derangement and hemodynamic modifications imposed to the heart by the no-flow state. It was

reasonable therefore to consider that a period of "restoring" CPR *before* attempting defibrillation could revert some of those processes that make the fibrillating heart refractory to shock.

Accordingly, a modification in the basic life support (BLS) protocol was introduced in the 2005 guidelines acknowledging that "recent evidence has suggested that a period of CPR before defibrillation may be beneficial after prolonged collapse" [9]. This evidence came from two clinical studies (one "before-after" study [10] and one randomized trial, RCT [11]) in which a period of 1.5-3 min of CPR before defibrillation improved outcome in the patients subgroup where emergency medical system (EMS) response time exceeded 4-5 min. Other experimental and predictive studies supported a positive role of CPR before defibrillation in long lasting VF (>5 min). However another RCT failed to prove benefit [12]. On this basis, it seemed reasonable to recommend "a period of about 2 min of CPR before defibrillation in patients with prolonged collapse (> 5 min)", but limiting this suggestion only to out-of-hospital CA and EMS protocols. No specific evidence was available for in-hospital CA.

More data were available to the 2010 ILCOR reviewers [13]. Another RCT [14] and other studies [15, 16, 17] did not show improvement in StHD, shifting the balance of evidence supporting CPR before defibrillation to a neutral equilibrium. Therefore, currently the "routine delivery of a specified period of CPR (e.g., 2-3 min) before rhythm analysis and shock is delivered, is no longer recommended". However if the protocol has already been implemented in local EMS following previous recommendations, "it is reasonable for them to continue this practice" [18] since it is not harmful.

Together with new guidelines, in 2010 two meta-analyses of available RCTs concluded that CPR before defibrillation did not demonstrate an improvement in terms of StHD and supported the equivalence of both approaches [19, 20], highlighting RCTs limitations (e.g., only the study of Baker et al. was sufficiently powered to detect difference in StHD). As shown in Table 1, RCTs were performed under different CPR protocols due to current guidelines (e.g., compression/ventilation ratio and number of consecutive shocks administered) and with local variations in response time. No clear data are available about quality of CPR administered from both bystanders and EMS personnel. Thus, studies with a larger number of patients and an univocal protocol were called for.

Recent clinical evidence: different designs, similar results.

In 2011, the Resuscitation Outcome Consortium (ROC) published the results of the "Early versus Delayed Analysis" (ROC PRIMED) trial [21]. The study had a large population sample (9933 patients), CPR protocol was standardized (30/2 ratio and single shock), EMS performances were carefully optimized by frequent retraining (every 6 months) and CPR quality was monitored. Furthermore, a more realistic protocol was designed instead of a difficult to define "defibrillation-first" versus "CPR-first" comparison. Indeed, it is common practice to

start CPR while the defibrillator is being retrieved even in patients in which shock is administered as soon as possible. In the ROC study, patients assigned to the "early-analysis" group received 30 to 60 seconds of CPR while the defibrillator was connected (i.e., before rhythm analysis); patients in the "late-analysis" group received 3 minutes of CPR before rhythm analysis. The StHD was exactly the same in the two groups (5.9%).Interestingly, in patients with the ideal situation of VT/VF as first recorded rhythm who had received bystander CPR, there was a tendency to decline in survival with delaying the rhythm analysis.

Another very recent RCT was performed in Taipei (Taiwan), where incidence of shockable rhythm as first-recorded rhythm is traditionally low (8%). A group of patients with out-of-hospital CA receiving a period of 10 cycles of 30:2 RCP (about 4 minutes) before rhythm analysis was compared with another group in which CPR and rhythm analysis were started together immediately after CA confirmation and defibrillation delivered as soon as VF/VT was detected. There were no differences in return of spontaneous circulation (ROSC) as primary outcome nor in StHD as secondary outcome. In a post-hoc analysis of ROSC patients, those receiving CPR before rhythm check had an higher rate of StHD [22].

To complete the picture, in a recent retrospective nationwide Japanese study , CPR before defibrillation did not show an improvement in long term outcomes compared with early defibrillation [23].

So far, in large human clinical studies CPR before defibrillation protocols have failed to demonstrate clear benefits in survival independently from the study design, local context, presumed CA interval, current compression/ventilation ratio and duration of CPR before shock. Is this the end of the road?

Table I. Characteristics of the main RCTs on Cardiopulmonary Resuscitation (CPR) *before* Defibrillation (Modified from: Meier et al. BMC Medicine 2010;8:52; and from: Simpson PM et al. Resuscitation 2010;81:925-931)

Rhythm analysis and defibrillation: the "depth bomb" model. As mentioned above, the rationale behind delaying the shock in favor of a period of compressions before defibrillation is to partially revert the processes imposed to the heart by the no-flow state. As described in Weisfeldt and Becker's 3-phase model [24], once VF has started, the heart passes through an immediate *electrical* phase lasting about 4 minutes in which defibrillation can be the sole intervention needed to restart the pump function, followed by about 10 minutes of *circulatory* phase in which compressions are required to increase the chance of successful defibrillation. Afterwards, the *metabolic* phase represents the time of irreversible changes with no effective treatment currently available (Figure 1).

Figure 1. The 3-phase model of Cardiac Arrest (CA). After ventricular fibrillation (VF) onset, the heart passes through three phases that require different treatments; VF waveform changes with passing of time (Adapted from: Weisfeldt ML, Becker LB. JAMA. 2002 Dec 18;288(23):3035-8).

Changes to the heart during this VF are multifactorial. Energy-substrate depletion induced by ischemia plays a role in the low response of the myocardium to defibrillation [25]. Hypoxia and acidosis increase the minimum defibrillation voltage (MDV) needed to convert VF in sinus rhythm highlighting the importance of recovering perfusion and oxygenation during CA [26]. The concept that VF is a high energy demanding condition is well established in cardiac surgery: during VF, oxygen consumption is 75% that of the beating working heart while subendocardial perfusion falls due to chambers distension that increases coronary resistance [27]. Indeed, as well as metabolic derangement, hemodynamic modifications must be taken into account. Elegant studies have described shape and volume changes in cardiac chambers during VF: the right heart overload leads to a bulging into the hollow left sections, decreasing the normal pressure gradient that drives coronary perfusion from aortic root toward coronary sinus. These seem early changes compared with metabolic alterations and could promptly be reversed by good compressions [28].

Currently, the extent of these modifications at the moment of rhythm analysis is impossible to be detect: when rescuers arrive at the victim's side, they can only confirm CA, start CPR and analyze the underlying rhythm.. In a setting in which even estimating CA onset interval can be difficult, they cannot understand the heart condition before CA nor which theoretical phase the heart is facing at the moment of their arrival. Furthermore, shockable rhythms are not all the same varying with underlying conditions and onset mechanisms [29]. Therefore, administering a shock can resemble dropping a depth bomb under a wavy surface while ignoring the actual characteristics of the target. Might we look for a specific radar?

Detecting signals of Ventricular Fibrillation: possible methods to identify the best timing for defibrillation.

The development of a non-invasive and real time monitoring that allows prediction of whether or not a shock would achieve ROSC would be important to prioritize rescuers' intervention (compressions or defibrillation?), reducing the number of failed defibrillation attempts and CPR interruptions. In fact, CPR pauses are mandatory for rhythm analysis prior to attempting defibrillation since compressions create artefacts on the ECG signal [30]. Even minimal interruptions (<4 secs) decreases myocardial perfusion to near 40% [31]. During pre-hospital CPR, pauses for rhythm analysis and defibrillation have been of more than 20 and 24 secs, respectively [32].

Established predictors of good quality CPR, i.e. coronary perfusion pressure and end-tidal CO_2 ($EtCO_2$),can predict defibrillation success [33]. *Coronary perfusion pressure* (the difference between minimal aortic pressure and right atrial pressure during compression diastole) is currently recognized as the best single indicator of the likelihood of successful defibrillation and ROSC [34]. However its invasive measurement during CPR is feasible in a very small minority of patients. *$EtCO_2$* is determined by body's CO_2 production and relationship between minute ventilation and pulmonary perfusion. Cardiac output produced by compressions is usually less than one-third of normal, reducing dramatically pulmonary flow and $EtCO_2$ [35]. $EtCO_2$ is highly correlated with coronary perfusion pressure during CPR, and may therefore serve as a tool for monitoring the effectiveness of compressions. When $EtCO_2$ exceeds the threshold level of approximately 10-15 mmHg, greater likelihood of successful ROSC has been reported [36]. However, practical measurements of $EtCO_2$ are currently limited by the need of endotracheal intubation.

ECG wwaveform analysis to predict defibrillation success

In contrast with CPP and $EtCO_2$, electrocardiogram (ECG) registered by external defibrillators is routinely available during CA. Therefore, ECG analysis of VF waveform waveformmight represent a non-invasive and real time approach to guide the priority of interventions and to predict the best timing for defibrillation delivery. There is evidence that VF waveform change over time. Several retrospective case series, animal studies, and theoretical models have suggested the possibility to predict the success of defibrillation by analyzing waveform. Indeed, VF features reflect vital organ blood flow and specifically myocardial blood flow and energy metabolism [37]. The story of this search began more than 25 years ago and included measurements of VF amplitude, first, and frequency, later.

'VF voltage', or signal *amplitude*, is defined as the maximum peak-to-trough VF amplitude in a given time window of the ECG signal [38]. Mean VF voltage is the average of VF voltage over the same time interval. It was observed that VF amplitude declines over time and greater amplitudes were associated with correspondingly greater defibrillation success. Weaver et al observed that, if VF amplitude was greater than 0.2 mV, likelihood of resuscitation was significantly greater [37]. VF voltage appeared not only as a predictor of ROSC but also as an indicator for timing the duration of VF following collapse.

Subsequently, it was realized that other parameters could be computed utilising the Fast Fourier transformation in a selected ECG interval. Brown et al developed a VF voltage and frequency analysis such as to obtain the so called '*median frequency*' (MF) which served as a predictor of successful defibrillation [39]. In a porcine model, a MF of more than 9.14 Hz had 100% sensitivity and 92% specificity in predicting the defibrillation success. MF was also correlated with coronary perfusion pressure in animal models as well as human victims and therefore it became the preferred ECG predictor of outcome [40]. In addition, MF appeared as a more accurate indicator for

estimating the duration of untreated VF, compared to the earlier VF amplitude. To improve sensitivity and specificity, more sophisticated methods of VF waveform analysis were investigated.

Amplitude Spectrum Area (AMSA)

Among different approaches, the Amplitude Spectrum Area (AMSA) is one of the most efficient ECG-derived defibrillation predictors in which mean amplitude and dominant frequency are combined. The amplitude spectrum is obtained by fast Fourier transform of the ECG scalar signal (Figure 2).

Figure 2. Fast Fourier Transformation with AMSA calculation.

In a porcine model, threshold values of AMSA for defibrillation success have been established and AMSA has confirmed its capability to optimize the defibrillation timing [41]. It was highly correlated with coronary perfusion pressure levels during CPR and significantly greater values were observed in animals that were resuscitated compared to those that were not [42]. AMSA demonstrated a negative predictive value for resuscitation of 96% and a positive predictive value of 78% both higher than other predictors, offering a guide to minimize ineffective shocks.

In clinical scenario AMSA values were significantly greater in successful defibrillation: a threshold value of 12 mV-Hz was able to predict the success of each defibrillation attempt with sensitivity and specificity of more than 91% [43].

The optimal duration of CPR before defibrillation may be difficult to define without an objective feedback measurement. It depends on the duration of VF and the quality of CPR, since poor myocardial perfusion is associated with decreased VF frequency spectrum. On the other hand, the increased VF amplitude and frequency indicate a positive effect of CPR with improved myocardial perfusion resulting in increased ROSC. The ECG and in turn AMSA analyses has the advantages of detecting changes in the electrical status of the myocardium during CPR [44]. More importantly, AMSA is not invalidated by artifacts resulting from compressions allowing for uninterrupted compressions during ECG analyses. Recently, Sun and colleagues [45], in a rat model of prolonged CA, optimized the duration of CPR and defibrillation timing by adopting a real time AMSA analyses during compressions. Shock was attempted only when AMSA achieved a predefined threshold level predictor of successful defibrillation: 70% of animals were resuscitated after the first shock in contrast to 0% when defibrillation was attempted following standard guidelines. The AMSA-guided CPR significantly improved ROSC success, reducing compressions interruptions and shock number prior to achieve ROSC and ultimately survival.

Recently we have investigated the accuracy of an AMSA-based defibrillation decision algorithm to guide CPR interventions in 1291 defibrillation events from 609 out-of-hospital VF patients [46]. AMSA value was significantly higher in successful defibrillation compared to unsuccessful ones (16.8 mV-Hz vs. 11.4, $p<0.0001$). More interestingly, with an AMSA threshold of 7.5 mV-Hz, a large amount of unnecessary defibrillations (35% for the first shocks and 50% for the subsequent shocks) were avoided with extremely high accuracy (Table II). Decreasing the threshold value, accuracy of AMSA in guiding shock decision further improved. An AMSA-based algorithm therefore clearly appeared as a useful approach to avoid many unnecessary and potentially harmful defibrillation in the clinical scenario.

Table II. Prediction of non successful defibrillation (defibrillation) attempts with an AMSA-based defibrillation decision algorithm.

Detecting different kind of VF

To improve prognostication, several studies have been focused on waveform changes in relationship to CA physiopathology, with more attention on CA as resultant of *ischemic* VF (i.e. the main cause of sudden death compared to experimental *electrically*-induced VF). It was unknown, in fact, whether waveform predictive utility differed in acute myocardial infarction (MI) or normal myocardium. AMSA changes and other VF features (i.e. AMSA slope), measured in animals with CA overlapping AMI or chronic ischemia, predicted ROSC independently of myocardial substrate. Furthermore, with compressions, the waveform evolved differently in the healthy or ischemic heart [47]. Thus, ECG predictors were, at least in part, related to the mechanism by which VF evolves and may distinguish an AMI-related CA.

Waveform analyses during CPR may also help in guiding the best treatment for *recurrent* VF and *shock-resistant* VF, the optimal treatment of which is still unknown. In out-of-hospital CA patients, AMSA and slope were higher in recurrent VF compared to shock-resistant VF, and recurrent VF was more likely to be defibrillated than shock-resistant VF. However, when VF was shock-resistant, AMSA and slope were highly predictive of defibrillation outcome [48].

Conclusions

Despite sensible background, the routinely application of CPR before defibrillation in clinical settings seems to not add the expected benefit in survival. Variables such as CA duration, underlying ischemic heart conditions, progressive hemodynamic changes, metabolic deterioration and quality of CPR affect the likelihood of defibrillation success. Currently, there are no simple instruction nor tools able to take in account all these variables while guiding CPR interventions. Promising evidence on ECG analysis have now emerged as a

clinically applicable method to provide a real-time indicator for compressions effectiveness and predictor of defibrillation success. The validity of VF feature analyses has been proven in animal studies and retrospective human trials. We therefore anticipate that these algorithms, incorporated into conventional defibrillators, will minimize interruptions in compressions and delivery of futile defibrillation, guiding to a more optimal timing of defibrillation. However, guidelines statements for clinical practice must be based on solid evidence. Unlikely, there are no prospective studies that have identified optimal VF waveform analyses algorithm as well as effects of medications and interventions (i.e. adrenergic blockade and hypothermia) on VF features. While awaiting for future diffusion of these promising smart technologies, early defibrillation in conjunction with high quality CPR, are the determinant weapons in the difficult battle to achieve resuscitation from CA.

Key messages

1. So far, clinical studies failed to clearly prove that an interval of CPR performed before attempting defibrillation can improve survival of cardiac arrest victims. Therefore, a specific period of CPR before defibrillation is no more recommended in current guidelines and defibrillation should be attempt as soon as possible with minimal interruptions in chest compressions.
2. Metabolic and hemodynamic cardiac changes occurring during ventricular fibrillation affect the possibility of successful defibrillation and may be reversed by chest compressions. Therefore, a real-time and non invasive tool able to detect heart responsiveness to defibrillation could be very useful to prioritize rescuers' intervention (compressions first or defibrillation first).
3. The ECG analysis and particularly the Amplitude Spectrum Area (AMSA) analysis has been proven to be a valid predictor of successful defibrillation in animal studies and retrospective human trials. AMSA can detect changes in the electrical status of the myocardium during CPR and it is not invalidated by artifacts resulting from compressions.

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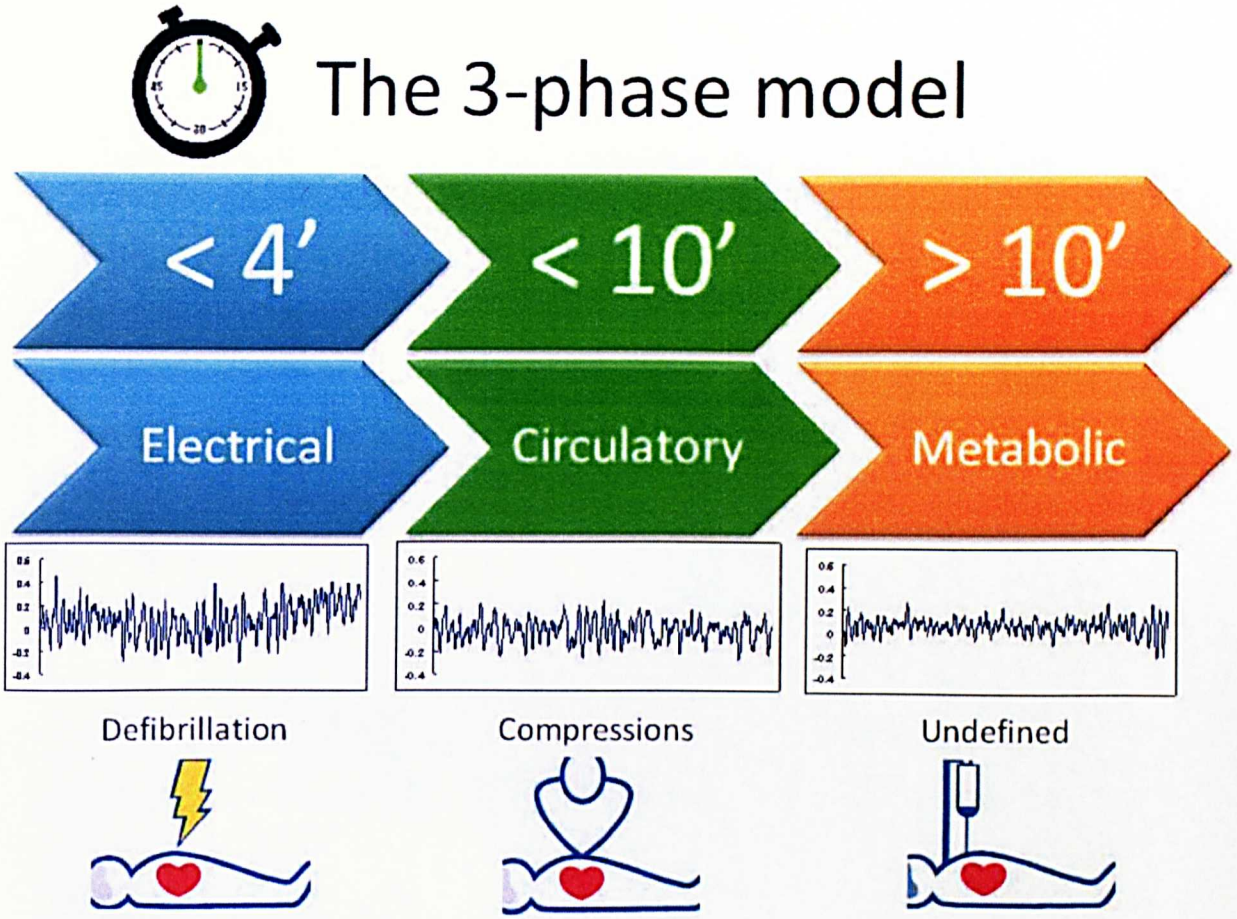
Table I. Characteristics of the main RCTs on Cardiopulmonary Resuscitation (CPR) *before* Defibrillation (DF)										
Authors	*Country*	*Publication (Year)*	*Sampling (Year)*	*Comparison*	*CPR first (sec)*	*Pts (n)*	*StHD (n, %) OR (95%CI) P value*	*C/V ratio*	*Shocks (n)*	*EMS mean response time (min)*
Wik et al.	Norway	2003	1998-2001	DF first vs CPR first	180	96 104	14 (15%) 23 (22%) 1.66 (0.80 to 3.46) P = 0.20	5:1	3	11.42 12.00
Jacobs et al.	Australia	2005	2000-2002	DF first vs CPR first	90	137 119	7 (5.1%) 5 (4.2%) 0.81 (0.25 to 2.64) P not provided	5:1	3	9.00 9.20
Baker et al.	Australia	2008	2005-2007	DF first vs CPR first	180	105 97	18 (17.1%) 10 (10.3%) 0.56 (0.25 to 1.25) P = 0.16	15:2 and 30:2	3 1	8.14 7.41
Jost et al.[49]	France	2010	2005-2008	DF first vs CPR first	60	424 421	45 (10.6) 56 (13.3) 1.27 (0.78-2.07) P = 0.34	CP*	3 vs 1	10.54 10.30
Stiell et al.	USA Canada	2010	2007-2009	Early vs Late analysis	30-60 vs 180	5290 4643	427 (8.1) 372 (8.0) -0.1 (-1.2 to 1.1) P = 0.92	30:2	1	6.00 6.00
MA et al	Taipei (Taiwan)	2012	2008-2009	DF first vs CPR first	240	148 141	11(7.4) 16 (11.3) 1.59 (0.71 to 3.57) p.= 0.31	30:2	1	6.70 6.70

C/V ratio: Compression to Ventilation ratio. EMS: Emergency Medical System. StHD: Survival to Hospital Discharge.
*Cardio-pump (Ambu, Denmark).
Modified from: Meier et al.(BMC Medicine 2010;8:52) and Simpson PM et al. (Resuscitation 2010;81:925-931).

Table II. Prediction of non successful defibrillation (DF) attempts with an AMSA-based defibrillation decision algorithm.

Selected AMSA thresholds	Events of correct prediction of 'non successful' DF	% of correct prediction overall 'non successful' DFs
First DF (n=609)		
5.5	124 (N=126)	98.4
6.5	175 (N=184)	95.1
7.5	213 (N=229)	93
Subsequent DFs (n=662)		
5.5	205 (N=210)	97.6
6.5	272 (N=283)	96.1
7.5	328 (N=346)	95



Adapted from: Weisfeldt ML, Becker LB. JAMA. 2002 Dec 18;288(23):3035-8.

Figure 1

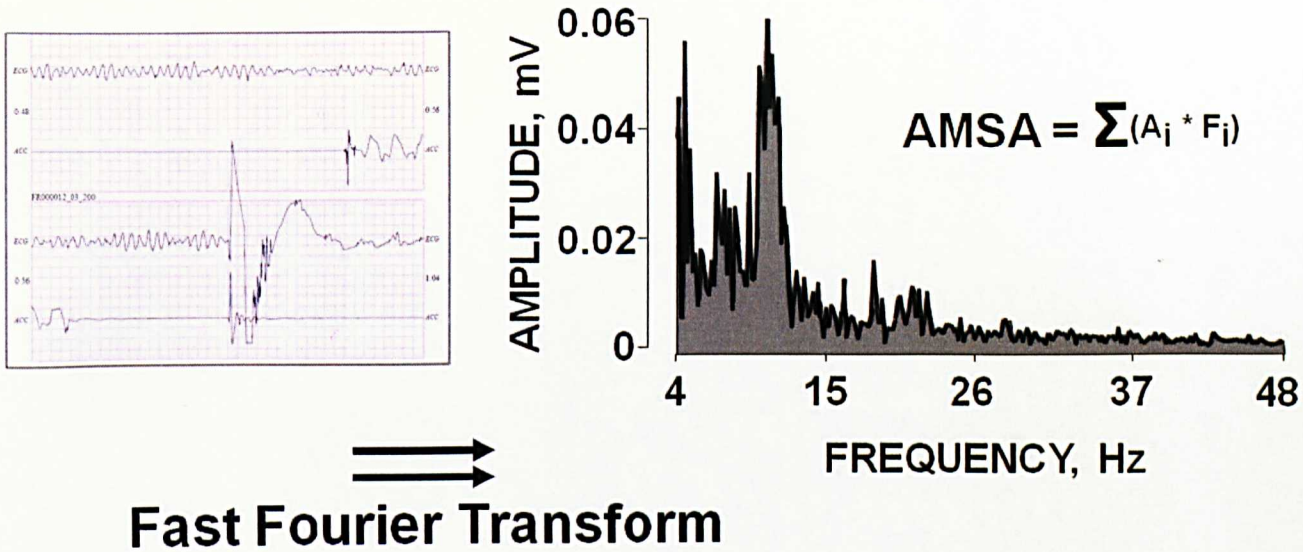


Figure 2

Metabolomics
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ORIGINAL ARTICLE

A combination of untargeted and targeted metabolomics approaches unveils changes in the kynurenine pathway following cardiopulmonary resuscitation

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Abstract The mechanisms responsible for post-resuscitation myocardial and cerebral dysfunction are not well understood, especially in the early post-resuscitation phases. In this investigation, we first adopted unbiased mass spectrometry-based metabolomic profiling to identify perturbations in circulating metabolites in a rat model of cardiac arrest and cardiopulmonary resuscitation. Our findings strongly indicated early alterations in a major route of the tryptophan catabolism, namely the kynurenines pathway, after resuscitation. Specific metabolites involved in the tryptophan catabolism were quantified absolutely using liquid chromatography-multiple reaction monitoring-mass spectrometry. Tryptophan plasma concentration fell significantly very early in the post-resuscitation phase, while its metabolites, L-kynurenine, kynurenic acid, 3-hydroxyanthranilic acid and 5-hydroxyindoleacetic acid, rose significantly. Changes in their concentration reflected changes in rat post-resuscitation myocardial dysfunction. Elevated plasma level of kynurenic acid, 3-hydroxyanthranilic acid were associated with significant decrease in ejection fraction and stroke volume. It is well known that kynurenines pathway is involved in the pathogenesis of numerous central nervous system disorders. By implication, altered levels of tryptophan metabolites in the early post resuscitation phase might contribute to the degree of cognitive recovery. Our results suggest that kynurenine pathway is activated early following resuscitation from cardiac arrest and might account for the severity of post-resuscitation syndrome. Our explorative investigation indicate that metabolomics can help to clarify unexplored biochemical pathways in cardiopulmonary resuscitation.

Keywords Cardiac arrest · Resuscitation · Plasma metabolomics · Kynurenines pathway · LC–MS · LC–MRM–MS

Abbreviations

3-HAA	3-Hydroxyanthranilic acid
5-HAA	5-Hydroxyanthranilic acid
5-HIAA	5-Hydroxyindoleacetic acid
CA	Cardiac arrest
CPR	Cardiopulmonary resuscitation
FFA	Free fatty acids
IDO	Indoleamine-2,3-dioxygenase
KYN	L-kynurenine
KYNA	Kynurenic acid
S1P	Sphingosine 1 phosphate
TRP	Tryptophan

1 Introduction

Novel metabolomics technologies have made it easier to acquire high-throughput snapshots of a whole organism's

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metabolic status and it is now possible to establish metabolic signatures in many relevant human diseases (Griffin et al. 2011; Locasale et al. 2012; Nordstrom and Lewensohn 2010). Knowing which metabolites are altered in a disease helps define its pathophysiology better. Metabolic profiling also facilitates high-throughput patient screening to diagnose the disease state or for risk assessment (Sabatine et al. 2005).

The application of metabolomic analysis in cardiovascular diseases is an emerging field and it is not yet possible to depict any single metabolic picture responsible for the prediction and progression of cardiovascular disease. However, the identification of clinically relevant changes in circulating metabolites is opening exciting avenues in the cardiovascular field (Abramson 1991; Alexander et al. 2011; Barderas et al. 2011; Lewis et al. 2008; Mayr et al. 2009; Rhee and Gerszten 2012; Turer et al. 2009; Wang et al. 2011b). For example, Shah and colleagues showed that a signature composed of dicarboxyacylcarnitines was predictive of further cardiovascular events in patients with coronary artery disease (CAD) and most significant differences persisted after adjustment for CAD risk factors (Shah et al. 2010). An important role of phospholipids as new key culprits in atherosclerosis was highlighted in patients with cardiovascular disease (Wang et al. 2011a)and a pathological role of ketone bodies has been suggested for human atrial fibrillation (Mayr et al. 2008). In acute ischemia and acute myocardial diseases many intermediates of the citric acid cycle were found to be depressed as a direct consequence of myocardial ischemia (Sabatine et al. 2005; Zhao et al. 2008).

Cardiovascular disease remains the main cause of death in developed nations and in Europe cardiac arrest (CA) is the leading cause of death (Chugh et al. 2004; Sans et al. 1997). The treatment for CA is cardiopulmonary resuscitation (CPR) to provide circulatory support, followed by defibrillation if a shockable rhythm is present. Morbidity and mortality after successful CPR largely depend on recovery of neurologic function. As many as 30 % of survivors of cardiac arrest, in fact, suffer permanent brain damage (Brown et al. 1992; Krause et al. 1986). The mechanisms responsible for post-resuscitation myocardial and cerebral injury are not well understood, although several events have been described. The reintroduction of oxygenated blood after the return of spontaneous circulation (ROSC) stimulates a sequence of complex actions that lead to acute inflammatory responses and release of reactive oxygen species (ROS), causing oxidative damage, cellular edema, cell membrane damage and apoptosis (Dezfulian et al. 2007; Lebuffe et al. 2003; Levraut et al. 2003; Ouyang et al. 1999). Several other processes, including interactions between pleiotropic mediators, coagulation abnormalities, activation of the inflammatory cytokine cascade, chemokine upregulation and ultimately recruitment of inflammatory leukocytes and reactive astrogliosis have also been reported after CA and are major players in the final outcome (Frangogiannis et al. 1998; Meybohm et al. 2009; Vakeva et al. 1998). There is still controversy about how much current biomarkers contribute to the information provided by conventional risk factors, and the clinical use of specific biomarkers (e.g. troponins, neuron-specific enolase, protein S-100 beta) to predict outcome of CA is still debated (Nolan et al. 2010). Predicting survival, myocardial and neurological outcome is therefore a difficult issue, especially in the early post-resuscitation phase.

The comprehensive quantitative assessment of plasma metabolites might help fill this information gap as metabolite differences in plasma provide the closest link to cellular metabolism in the whole body and its response to resuscitation. Thus, we hypothesized that extensive characterization of the largest possible number of metabolites from relevant or potentially affected metabolic pathways might help in identifying mechanisms explaining the outcome of CA and CPR and might serve as early prognosticator biomarkers and ultimately as targets for therapeutic intervention.

The aim of this study was to examine plasma metabolites in a rat model of CA in post-resuscitation observational periods to identify perturbation in circulating metabolites and thus potential mechanisms accounting for outcome of cardiac arrest. We used a well-known rat model of CA and CPR (Sun et al. 2010). Changes in plasma metabolites in the early post-resuscitation phase were identified using an untargeted metabolomics approach by liquid chromatography/tandem mass-spectrometry (LC–MS/MS). On the basic of the findings, specific metabolites involved in the tryptophan metabolism were then targeted, using LC-multiple reaction monitoring (MRM)-mass spectrometry.

2 Materials and methods

2.1 Standards and chemicals for metabolomic analysis

HPLC-grade acetonitrile, ammonium acetate (>99 % dry matter) and formic acid (98 %) were purchased from Fluka (Buchs, Switzerland). HPLC grade MilliQ water was obtained with a MILLI-RO PLUS 90 apparatus (Millipore, Molsheim, France). Analytical standards L-tryptophan (TRP), L-kynurenine (KYN), kynurenic acid (KYNA), 3-hydroxyanthranilic acid (3-HAA), 5-hydroxyanthranilic acid (5-HAA), 5-hydroxyindoleacetic acid (5-HIAA) were from Sigma–Aldrich (Italy). Deuterated standards D8-L-tryptophan, D6-kynurenic and D5-5-hydroxyindoleacetic acid were from CDN Isotopes (Chemical Research 2000 S.r.l., Italy). D4-L-kynurenine and D2-3-hydroxyanthranilic

acid were from Buchem BV (Netherlands). Individual stock solutions were prepared in MilliQ water and stored at −20 °C. Working solutions containing all the metabolites and their internal standards were prepared freshly before analysis.

2.2 Rat model of cardiac arrest

Procedures involving animals and their care conformed to institutional guidelines in compliance with national (4D.L. N.116, G.U., supplement 40, 18-2-1992) and international (EEC Council Directive 86/609, OJ L 358, 1, 12-12-1987, National Institutes of Health's *Guide for the Care and Use of Laboratory Animals*, and US National Research Council 1996) law and policies. All efforts were made to minimize the number of animals used and their suffering.

An established rat model of CA and CPR was used. Eighteen male Sprague–Dawley rats weighing 470 ± 25 g were anesthetized with pentobarbital, endotracheally intubated and surgically instrumented, as previously described (Sun et al. 2010). Ventricular fibrillation (VF) was induced in 12 rats by delivering up to 4 mA AC current into the right ventricle. CPR, including mechanical chest compression, ventilation with oxygen, and epinephrine (0.02 mg/kg), was then started and continued for a 6 min before defibrillation. Chest compression was maintained at a rate of 200/min with equal compression–relaxation and ventilation at 50/min. Resuscitation was attempted with up to three two-joule counter-shocks. Animals were considered successfully resuscitated if supra-ventricular rhythm returned with mean aortic pressure above 50 mmHg. Two ($n = 6$) and 4 h ($n = 6$) after resuscitation, animals were anesthetized and surgically prepared for femoral artery cannulation for blood withdrawal. Animals were then euthanized. Blood was collected into EDTA-tubes and centrifuged for 10 min at 2,000×*g* at room temperature. Plasma samples from each animal were immediately stored at −80 °C. Six other rats were not subjected to CA and served as controls (CTR). Body temperature was held at 37 ± 0.5 °C throughout the experiment.

2.3 Metabolomic profiling by LTQ-Orbitrap mass spectrometry

Discovery pilot metabolomics analysis began with an unbiased search for plasma analytes linked to post-resuscitation using two experimental groups from the rat model. The *control group* (CTR, $n = 3$) was selected from rats not subjected to CA, while the *case group* was selected from rats subjected to CA and CPR and sacrificed 2 h after resuscitation (CA/CPR2, $n = 3$, early post-resuscitation phase). To reduce biological variation that might mask important changes in metabolite abundances, we prepared metabolite samples by pooling plasma (5 µL/animal) from individual rats in the experimental groups. Every specimen made an equal contribution to the pool and two composite groups (CTR and CA/CPR2) were then created.

Metabolites were extracted by adding four volumes of cold methanol to the plasma sample; samples were vortexed and incubated at −20 °C for 1 h. They were then centrifuged 10 min at 14,000×*g*, and the supernatant (rich in small-molecules analytes) was collected, dried in a SpeedVac and resuspended in 20 µL of 0.1 % formic acid.

A portion (2 µL) of metabolite extract from the CTR and CA/CPR2 groups was directly analysed by LC–MS/MS, using an LTQ Orbitrap XL™ (Thermo Scientific, Waltham, MA, US), interfaced with a 1,200 series capillary pump (Agilent, Santa Clara, CA, US). The MS instrument was operated in positive (POS) and negative (NEG) ionization modes. Analyses were run in triplicate.

Metabolites were separated on an Agilent Technologies Zorbax C18 SB column (150 × 0.5 mm ID, particle size 5 µm). Flow rate 10 µL/min with mobile phases: water containing 0.1 % formic acid (A) and acetonitrile (B) for the positive ion. For negative ion mode, 2 mM of ammonium acetate was substituted for the 0.1 % formic acid. The gradient consisted of 5 % B for 5 min, followed by a linear gradient to 95 % B over 45 min, hold at 95 % B for 5 min, and re-equilibration at 5 % for 2 min.

MS conditions were: source DESI Omni Spray (Prosolia, Indianapolis, IN) used in nanospray mode with positive and negative ion modes; ion spray voltage 2,100 V; capillary temperature 220 °C; capillary voltage, 42 V. MS spectra (*m/z* 100–1,000) were acquired in the Orbitrap analyzer at 60,000 resolution, in parallel with the low-resolution MS/MS scans of the four most abundant precursor ions being acquired in the LTQ. The lock-mass option was used to obtain the most accurate mass measurements in MS mode. The polydimethylcyclosoloxane ion generated in the electrospray process from the ambient air (protonated $(Si(CH_3)_2O)_6$, *m/z* 445.120025) was used for internal recalibration in real time. MS/MS analysis was done in data-dependent mode (DTA) using Xcalibur software (Thermo Scientific, Waltham, MA, US) with target ions previously selected for the MS/MS dynamically excluded for 30 s.

To ensure the stability and repeatability of the LC–MS systems, ten runs of pooled samples were done on the system before the sample run sequence. Samples were run in an order that alternated the CTR and CA/CPR2 groups to reduce any systematic error associated with instrumental drift.

2.4 Untargeted metabolomics data processing and statistical analysis

All LC–MS files were analyzed using the MS label free differential analysis software SIEVE v1.3 (ThermoFisher, Cambridge, MA, US). SIEVE was run on all the LC–MS full-scan chromatograms using the *small molecule* setting. The chromatograms were time-aligned, referencing the CA/CPR2 sample acquired in the middle of the sequence. The framing parameters were set at 0.01 Da for the *m/z* window and 0.35 min for the retention time (RT) window; 500,000 was used as the intensity threshold. Prior to performing any statistical analysis, an additional filtering criteria was applied to include in the dataset only frames with an intensity coefficient of variation (CV%) <10. The preprocessed results were then fed into the SIMCA-P 13 (Umetrics, Umea, Sweden) platform for multivariate analysis. Principal component analysis (PCA) was performed on intensity data, preprocessed using the Pareto scaling, to examine cluster and outliers within the observations. All analyses were performed on data from both ion modes separately. Univariate analysis was performed using a 2-tailed Welch *t* test ($p < 0.01$; Prism v. 5.0; GraphPad Software Inc, USA) to identify metabolites presenting intensities significantly different in the two experimental groups.

2.5 Identification of plasma metabolites

For metabolite identification, the frame *m/z* values were used for batch searches on the METLIN database (http://metlin.scrpss.edu) and Human Metabolome Database (HMDB, http://www.hmdb.ca/). Both sites allow the user to search by ionization mode, either positive or negative. Accurate mass data and isotopic distribution for the precursor and product ion were compared to spectral data of the reference compounds in the databases. Definite identifications were reported only for metabolites with accurate mass match <5 ppm.

2.6 Mapping metabolic pathways

For biological interpretation of the metabolite dataset by our untargeted strategy, we mapped the identified metabolites to the KEGG pathway database (Kyoto Encyclopedia of Genes and Genomes; (www.genome.jp/kegg/), using MetaboAnalyst 2.0, a comprehensive online tool suite for metabolomic data analysis and interpretation (www.metaboanalyst.ca). Metabolite sets were analyzed to identify biologically meaningful patterns that were significantly enriched in our metabolomic data. The OverRepresentation Analysis (ORA) algorithm was applied and the hypergeometric test was used to see whether a particular metabolite set was represented more than expected by chance in the given compounds list. Then the Pathway Analysis Module was used to combine the enrichment analysis results with the pathway topology analysis (centrality measures to estimate node importance) to identify the most important pathways involved in early CPR (Xia and Wishart 2011).

2.7 Absolute quantification of plasma tryptophan metabolites by LC-multiple reaction monitoring (MRM) coupled with isotope-dilution mass spectrometry

Plasma (20 µL) from each animal ($n = 6$) in each experimental group (CTR and CA/CPR2, CA/CPR4,: respectively 2 and 4 h after resuscitation, respectively) was spiked with 10 µM of deuterated standards (tryptophan-D8, L-kynurenine-D4, kynurenic acid-D5, 3-hydroxyanthranilic acid-D2, 5-hydroxyindoleacetic acid-D5). Spiked plasma samples were then deproteinized by mixing with four volume of cold methanol, vortexed, and incubated at −20 °C for 1 h. Samples were centrifuged 10 min at 14,000×*g*, the supernatant was collected, and the centrifugation was repeated. The supernatant was dried in a SpeedVac and resuspended in 20 µL of 0.1 % formic acid. 10 µL of supernatant were analysed directly by LC–MS/MS with the Agilent 1200 series system for LC. Separation was with a Synergy 4u Fusion-RP 80A column (50 × 2.00 mm, Phenomenex) using as mobile phase A 0.1 % formic acid in water and mobile phase B 100 % acetonitrile at a flow rate of 0.2 mL/min. Elution started with 99 % of A and 1 % of B, followed by a 13-min linear gradient to 99 % of B, a 2-min isocratic elution and a 1-min linear gradient to 99 % of A, which was maintained for 8 min to equilibrate the column.

The mass spectrometric analysis was done using an Agilent 6410 triple quadrupole mass spectrometer (Agilent Technologies) in positive ion mode for all the metabolites. Typical chromatograms from the analysis of rat plasma are presented in supplementary Fig. S1. Instrumental conditions optimized for each compound are summarized in supplementary Table S1. Quantitative analyses were processed with MassHunter workstation quantitative analysis software v B.01.04 (Agilent Technologies).

2.8 Performance of the quantification method

The performance of the method was assessed in rat plasma samples for all the TRP metabolites. Recoveries and repeatability were assessed by analyzing rat plasma samples in five replicates. Since plasma already contained substantial amounts of metabolites, the samples were spiked with 1 µM of KYN, KYNA, 3-HAA, 5-HAA, 5-HIAA and 180 µM of

TRP before extraction and processed as previously mentioned. Known amounts of isotopologues (10 pM) were then added to samples before LC–MS/MS analysis. Blank samples (water + 0.1 % formic acid) were analyzed in each analytical run to test and correct bias. Instrumental quantification limits (IQL) were determined by directly injecting standard solutions with increasing amounts of each metabolite. The limits of quantification (LOQ) for the whole method were calculated directly from extracted samples as the concentrations giving peaks with a signal-to-noise ratio of 10. The linearity of the calibration curves was tested in the concentration ranges normally measured in rat plasma and a calibration curve was injected during each analytical run to check the linearity (correlation factors) and the instrumental repeatability. Intra- and inter-day instrumental repeatability and precision were also assessed by replicated injections of standard mixtures and rat plasma samples.

2.9 Correlation with hemodynamic and myocardial functions

We assessed whether the relative levels of individuals TRP's metabolites correlated with post resuscitation myocardial dysfunction developed in each rat, following ROSC by using the Pearson correlation coefficient (r) (SPSS 16.0).

2.10 Targeted metabolomics statistical analysis

Physiological parameters in the rat model and metabolite concentrations in different experimental groups were compared by one-way ANOVA followed by a multiple comparison test (Tukey–Kramer *HSD* test) computed using Prism v. 5.04 (GraphPad Software Inc, USA). The limit of statistical significance was set at $p < 0.05$.

3 Results

3.1 Rat model of cardiac arrest and experimental measurements

Some key measurements of the rat model of CA used are summarized in Table 1. There was no differences in the body weight of the animals and in their heart rate values. 2 and 4 h after CPR, there was a significant decrease in value of mean arterial pressure, coronary perfusion, ejection fraction, cardiac output and stroke volume compared to controls. More details are reported in Ristagno et al. 2012, in preparation.

Table 1 Experimental measurements, hemodynamics and myocardial functions in the rat model of cardiac arrest

	CTR	CPR2h	CPR4h
Body weight (g)	468 ± 35	480 ± 24	471 ± 16
Heart rate (beats/min)	387 ± 35	357 ± 19	377 ± 16
Mean arterial pressure (mmHg)	136 ± 2	105 ± 2*	105 ± 5*
Coronary perfusion pressure (mmHg)	116 ± 3	87 ± 2*	91 ± 4*
Ejection fraction (%)	79 ± 2	42 ± 2*	48 ± 4*
Cardiac output (mL/min)	122 ± 10	74 ± 7*	66 ± 5*
Stroke volume (mL)	0.34 ± 0.02	0.21 ± 0.02*	0.18 ± 0.02*
Declaration time (ms)	28 ± 4	18.8 ± 1.2	19.4 ± 1.3
End diastolic volume (mL)	378 ± 24	446 ± 51	465 ± 57

Data are mean ± SD ($n = 6$). Asterisks mark significant expression differences from CTR (one-way ANOVA, Tukey–Kramer HSD, * $p < 0.01$)

CTR rats not subjected to cardiac arrest as control, *CPR2h and CPR4h* rats subjected to cardiac arrest and cardiopulmonary resuscitation, euthanized respectively 2 and 4 h after resuscitation

3.2 Plasma metabolome profiles in the early post-resuscitation phase

To initially explore the metabolome changes associated with the early post-resuscitation phase, a plasma LC–MS/MS approach was used to discover unbiased small-molecule metabolic profiles in rats not subjected to CA (CTR) and in rats subjected to CA and CPR and sacrificed 2 h after resuscitation as described in Methods. Mass-spectral data were subject to peak alignment and data pre-processing by SIEVE 1.3. Then, data were analyzed for global changes by using multivariate statistics to determine group separation as well as univariate statistics to evaluate the number and percentage of features that vary significantly between the two sample sets. As seen in Fig. 1, PCA (principal component 1 vs principal component 2) revealed an excellent separation of the two experimental groups under both positive and negative modes.

Up to 4,534 and 4,710 features respectively were detected in positive and negative ion mode. 1,306 features were detected in both sample sets, showing significant changes in their relative signal intensity (defined as a ≥twofold change with CV% ≤10 and Welch's t test $p \leq 0.01$,) (Supplementary Table S2). Of these 1,306 features, 141 were related to molecular species identified by database searches (METLIN and HMBD) and are listed in Supplementary Table S3. It should be noted that a given molecule may be represented by several different features, such as naturally occurring components of its isotopic

Table 2 Significant correlation among plasmatic levels of tryptophan and its metabolites with hemodynamic and myocardial functions

	Pearson r-coefficient	*p* Value
Tryptophan vs		
Ejection fraction	0.546	0.016
Cardiac output	0.492	0.032
Stroke volume	0.596	0.009
Kynurenic acid vs		
Ejection fraction	−0.771	0.0001
Stroke volume	−0.625	0.013
3-Hydroxyanthranilic acid vs		
Ejection fraction	−0.641	0.004
Cardiac output	−0.632	0.005
Stroke volume	−0.639	0.006
5-Hydroxyindolacetic acid vs		
Ejection fraction	−0.691	0.002

Pearson correlation coefficient (r) was assessed by SPSS 16.0

cluster or non-specific adduct ions. Several analytes were detected only in positive mode, while others were observed only in the negative ion mode as already reported for plasma samples (Nordstrom et al. 2008). To further interpret the biological significance in the early post-resuscitation phase, we used MetaboAnalyst tools to link these metabolites to metabolic pathways.

Figure 2 shows the results of the metabolic pathway' analyses. Panel A gives the summary plot for the metabolite set enrichment, panel B shows the difference in abundance of the metabolites, mapped into enrichment categories, and panel C shows all metabolic pathways arranged according to the scores from enrichment analysis (*y* axis) and topology analysis (*x* axis). Analysis of the differences between the two datasets indicated that the beta-oxidation of fatty acids, linolenic acid metabolism, TRP metabolisms and sphingolipid metabolism were over-represented (Fig. 2, panel A). The very early CPR phase (2 h) showed plasma changes, although modest, in the composition of free fatty acids (FFA) compared to control. For example there was a generally lower level of unsaturated FFA (*e.g.* linoleic, acid, docosahexaenoic acid) and saturated acids (*e.g.* capric, myristic and dodecanoic acids). Rats from the early CPR interval also had lower plasma levels for sphingolipids such as sphiganine-1-phosphate and sphingosine 1-phosphate. Interestingly, striking differences from controls were seen in various intermediates of TRP catabolism through either kynurenine or a series of indoles in the early post-resuscitation phase (*e.g.* low level of indoxyl; higher levels of 5-hydroxyindolacetic acid, formyl-5-hydroxykynurenamine, 4-(2-aminophenyl)-2,4-dioxobutanoate, KYNA, indolelactic acids) (Fig. 2, panel B). Pathway analysis (Fig. 1, panel C) showed that the TRP metabolism was more likely to be significantly perturbed as a consequence of early CPR and, depending on the location on the plot, changes in the metabolites might have an impact on the pathway as inferred from pathway analysis reported in Supplementary Table S4.

3.3 Absolute quantitation of circulating tryptophan metabolites

Because TRP metabolism was one of the most important pathways perturbed during the early post-resuscitation phase, we explored whether the major route of TRP catabolism, the kynurenhine pathway (KP) and the TRP metabolism to serotonin metabolite, played a role throughout a longer post-resuscitation observational period of prolonged duration. Figure 3 gives a schematic view of the TRP catabolism.

We developed a highly sensitive and specific isotope dilution LC–MS/MS method for accurately quantifying tryptophan (TRP), L-kynurenine (KYN), kynurenic acid (KYNA), 3-hydroxyanthranilic acid (3-HAA), 5-hydroxyanthranilic acid (5-HAA), 5-hydroxyindoleacetic acid (5-HIAA) in rat plasma at 2 and 4 h after resuscitation, compared to controls. Deuterated metabolites were added to plasma samples as internal standards.

We assessed the assay performance in terms of linearity, sensitivity and analytical recovery. The instrumental sensitivity was good and the IQLs ranged from 0.0015 to 0.25 pmol/injected. LOQs in rat plasma ranged between 0.55 and 6 nM, except for 5-HIAA (40 nM) (Supplementary Table S5). The recoveries in rat plasma were higher than 60 % for all the metabolites (Supplementary Table S5). The analytical response was linear for all the compounds in the range of concentrations measured in rat plasma and the inter-day correlation factors (r2) were $\geq$0.9994 with standard deviations (SD) $\leq$0.0007 (Supplementary Table S6). Instrumental repeatability, assessed using replicate injections of standard mixtures and rat plasma, was generally $\leq$10 % except for 5-HAA that ranged from 10.6 to 17.2 % (Supplementary Table S6).

Figure 4 shows the mean rat plasma concentrations of TRP and its metabolites KYN, KYNA, 3-HAA, 5-HAA and 5-HIAA measured over the intervals after resuscitation in each of the six rats per group. Plasma levels in rats not subjected to CA were taken as the baseline concentration.

Significant changes in plasma concentrations of TRP and the metabolites showed significant differences from controls 2 and 4 h after resuscitation. TRP decreased significantly at both times (Tukey HSD $p < 0.05$). Interestingly, the concentrations of KYN, KYNA and 5-HIAA almost doubled in the 2 h after resuscitation (Tukey HSD $p < 0.05$) and then dropped back to baseline towards 4 h, although only KYNA and 5-HIAA reached significance

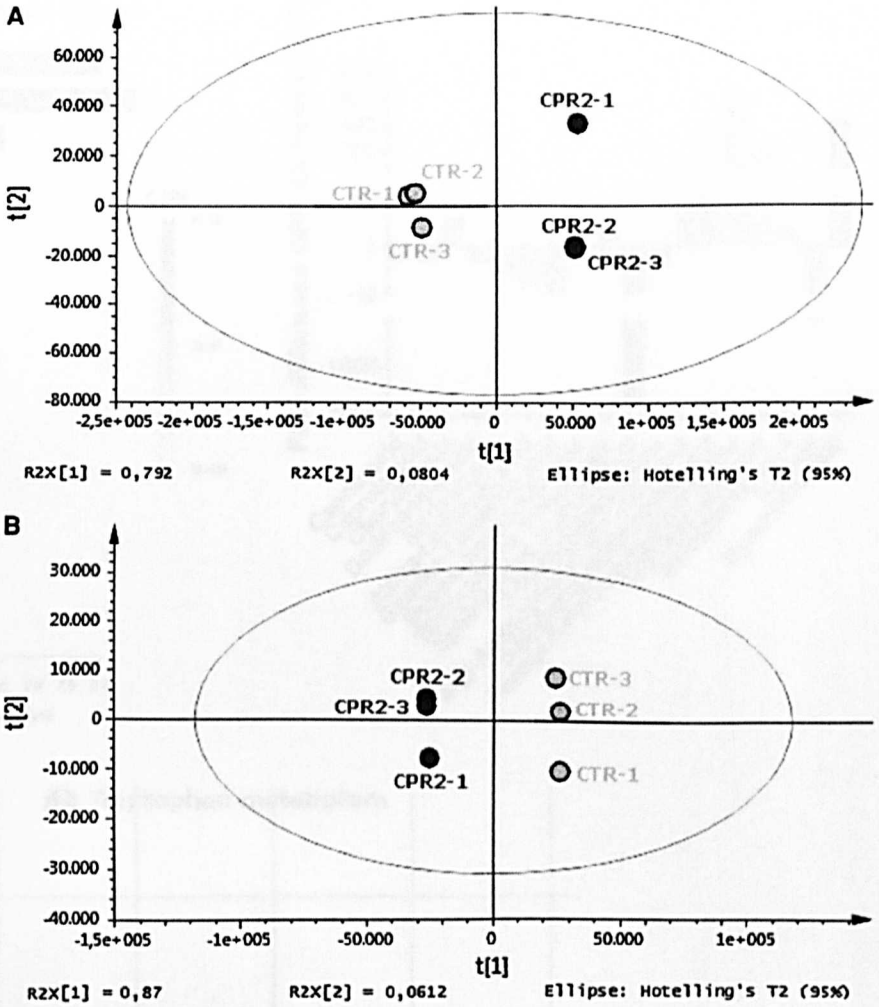

Fig. 1 PCA score *plots* of the plasma profiling of rats not subjected to cardiac arrest as control (CTR) and rats subjected to cardiac arrest and cardiopulmonary resuscitation, euthanized 2 h after resuscitation (CPR2h). *Panel A* with the positive ESI dataset. *Panel B* with the negative ESI dataset (see "Materials and methods" section)

between 2 and 4 h after CPR. The exception was 3-HAA, whose concentration rose significantly at 2 h and was still significantly higher than baseline at 4 h. 5-HAA did not significantly differ from baseline at any time. As reported in Table 2, changes in plasmatic levels of TRP, KYNA, 3-HAA and 5-HIAA were significantly correlated with different echocardiographic data. For example, the decrease in ejection fraction, cardiac output and stroke volume observed in animal following ROSC was inversely related with the increase in 3-HAA plasmatic level, but directely related with TRP concentration.

4 Discussion

Emerging metabolome profiling technologies offer the possibility of identifying novel biomarkers and pathways activated in cardiovascular diseases; however, applications to post-resuscitation myocardial dysfunction in the setting of CA are still lacking. We used a global LC–MS metabolomics approach to obtain a comprehensive view of changes in plasma metabolites associated with CPR in an established, widely accepted rat model.

We first adopted an unbiased strategy towards profiling as many plasma metabolites as possible in the very early post-resuscitation phase (CPR 2 h) after CA in rats. The profiling data was then used to detect altered biochemical processes using bioinformatics-based pathway mapping. Statistically significant signatures can be obtained in rats plasma in the very early CPR period compared to control rats at baseline. Many biochemical alterations were in line with those already reported in the literature about cardiovascular dysfunctions, supporting the feasibility and robustness of our explorative untargeted LC–MS strategy. For example, there were changes in the plasma levels of some saturated and unsaturated fatty acids (FA), with an overall tendency to decrease in the early CPR phase. This de-regulation have significant energetic and functional consequences on the heart, affecting the delivery of FFA to the myocardium and their utilization during ischemia and

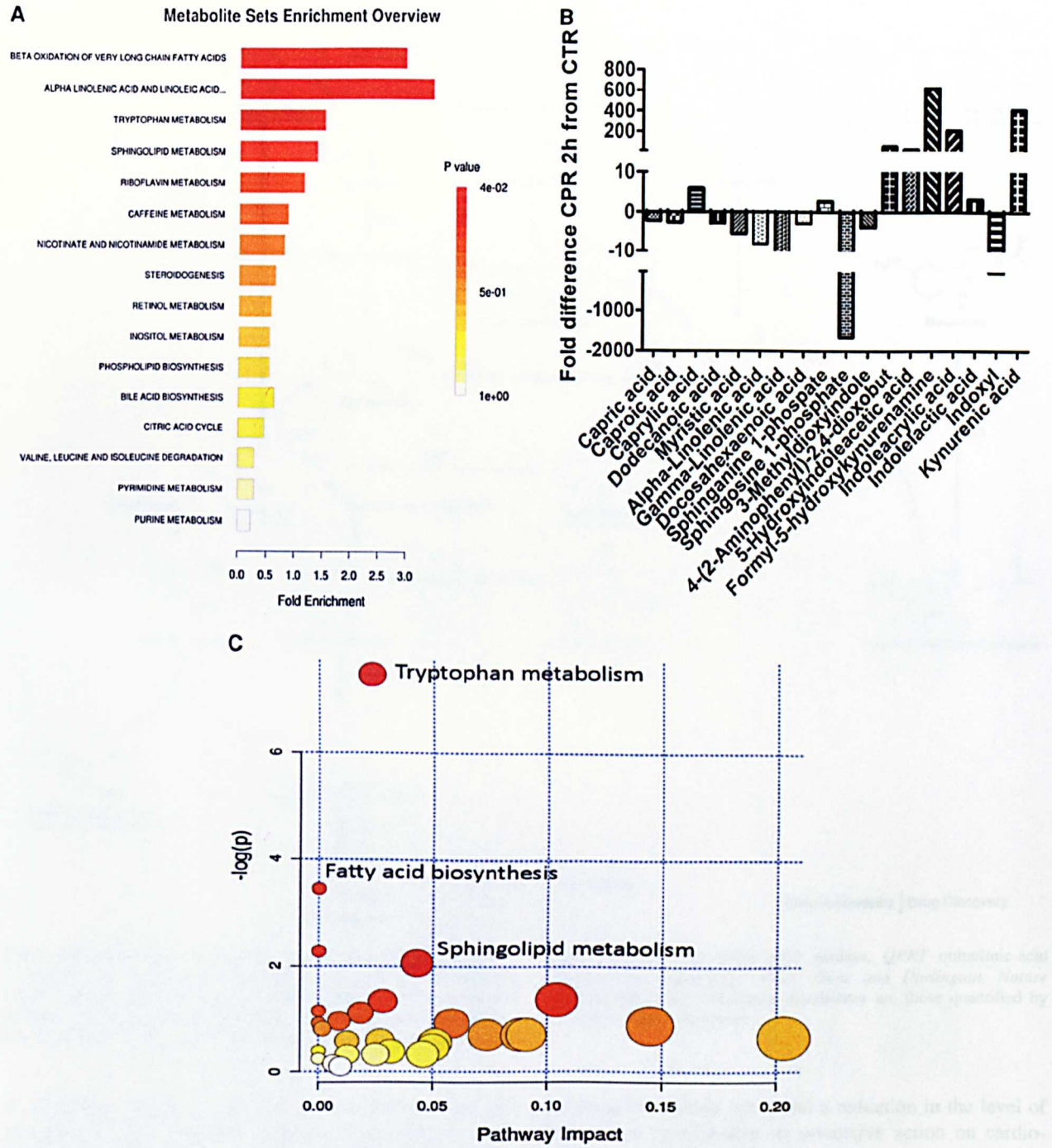

Fig. 2 Metabolic pathway analyses related to the metabolites that specifically differ in the two datasets (rat CTR and rats CPR2h) utilizing the MetaboAnalyst functional interpretation tools. *Panel A* graphic summary of metabolite set enrichment analysis; the *horizontal bars* summarize the main metabolite sets identified in this analysis; the *bars* are *colored* based on their *p* values and the *bar length* is based on the -fold enrichment. *Panel B* shows the difference in abundance of the metabolite subset mapped into the enrichment categories. *Panel C* shows all metabolic pathways arranged according to enrichment analysis (*y* axis) and topology analysis (*x* axis) scores (see "Materials and methods" section)

the post-ischemic period. The involvement of FA beta-oxidation in rats in the early CPR phase is consistent with the decrease in the myocardial capacity for this process in rodent models of heart failure (Lopaschuk et al. 2010) and in clinical settings, as the rates of FA uptake and oxidation were low in patients with dilated cardiomyopathy (Jaswal

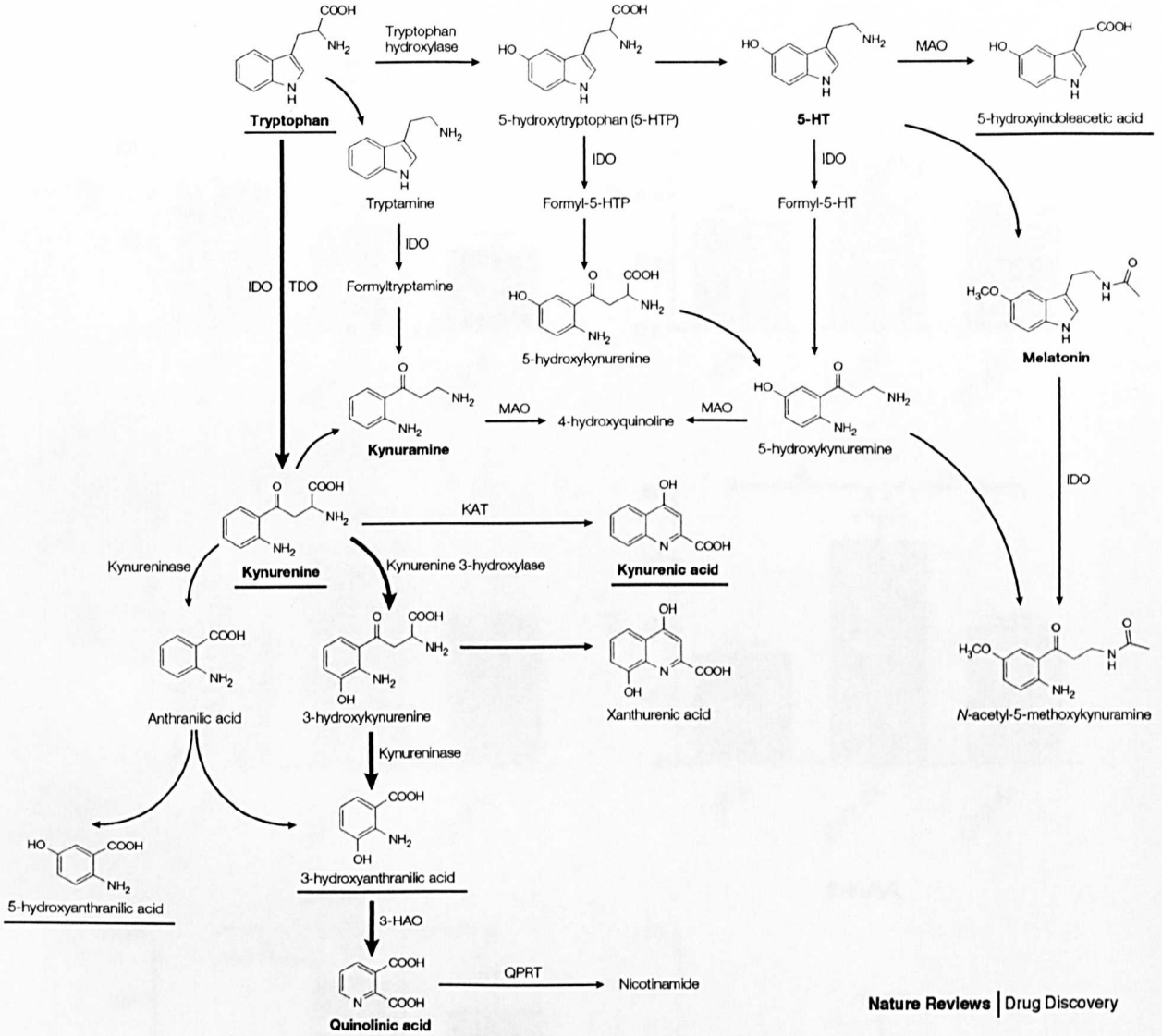

Fig. 3 Key elements of the tryptophan breakdown metabolic pathway. Conversion of tryptophan to 5-hydroxytryptamine (5-HT) and to 5-hydroxyindolacetic acid and tryptophan oxidation by the kynurenine pathway. *IDO* indoleamine 2,3-oxidase, *TDO* tryptophan 2,3-dioxygenase, *MAO* monoamino-oxidase, *KAT* kynurenine aminotransferase, *3-HAO* 3-hydroxyanthranilic acid oxidase, *QPRT* quinolinic-acid phosphoribosyl transferase. From *Stone and Darlington, Nature Reviews*, 2002. The *underlined* metabolites are those quantified by LC–MRM–MS in this study

et al. 2011; Neglia et al. 2007). Our findings are in accordance with a general reduction in oxidative fuel metabolism and greater reliance on anaerobic metabolism for energy revealed by metabolomics analysis (Turer et al. 2009) in clinical settings of myocardial ischemia/reperfusion.

There is convincing body of the role of sphyngolipids in cardiovascular dysfunctions (Knapp 2011) and in particular for S1P, which has potent cardioactive properties against ischemia–reperfusion injury (Baranowski and Gorski 2011). In line with these observations, in rat plasma from the early CPR phase, we found a reduction in the level of S1P, which could lessen its protective action on cardiomyocyte viability (Knapp et al. 2009).

Analysis of rat plasma using a comprehensive LC–MS platform also identified metabolites of the essential amino acid TRP catabolism that had not, to our knowledge, been previously associated with any post-resuscitation syndrome. We identified what we believe to be novel changes in plasma after CPR.

The targeted LC–MRM–MS platform with stable-isotope dilution method enabled us to unambiguously quantify

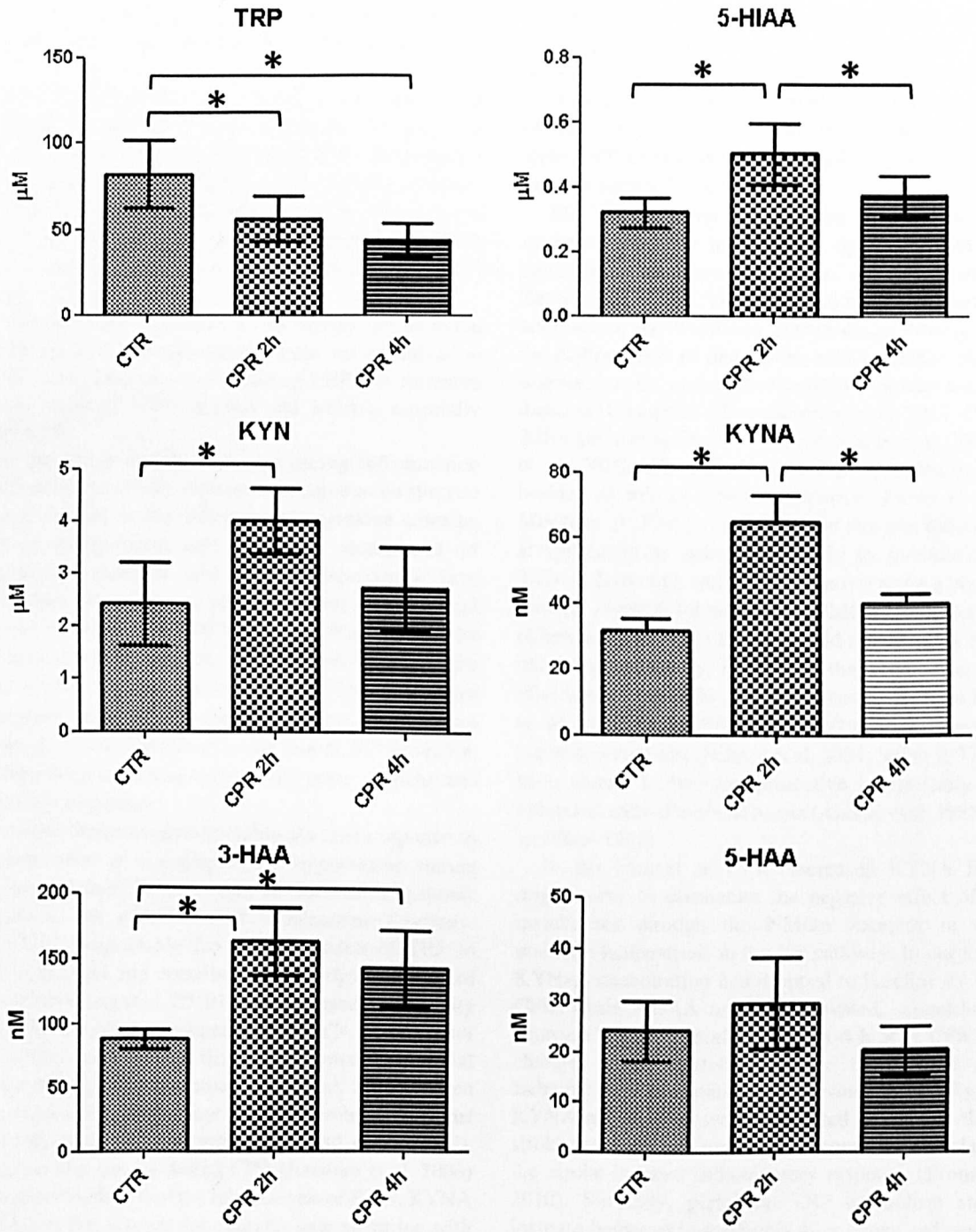

Fig. 4 Rat plasma concentrations of tryptophan and its metabolites at different cardiopulmonary resuscitation phases (CPR). Each *bar* represents the metabolites' plasma concentration as mean ± SD (n = 6 rats/group). *Asterisks* mark significant differences in expression (one-way ANOVA, Tukey–Kramer HSD, $p < 0.05$). *CTR* rats not subjected to cardiac arrest, as control, *CPR2h and CPR4h* rats subjected to cardiac arrest and cardiopulmonary resuscitation, euthanized respectively at 2 and 4 h after resuscitation, *TRP* tryptophan, *KYN* L-kynurenine, *KYNA* kynurenic acid, *3-HAA* 3-hydroxyanthranilic acid, *5-HAA* 5-hydroxyanthranilic acid, *5-HIAA* 5-hydroxyindoleacetic acid

the statistically meaningful changes of TRP and its metabolites throughout the post-resuscitation observational periods. The TRP metabolites analyzed mapped along two distinct routes: (i) the methoxyindoles pathway that regulates the conversion of TRP into the neurotransmitter serotonin and (ii) the kynurenine pathway (KP), through

which TRP is metabolized towards neuroactive kynurenines and nicotinamide adenine dinucleotide (NAD) (refer to Fig. 2).

Methodological refinements, including optimization of MS conditions, and deuterated standards resulted in a sensitive, selective and accurate method for the simultaneous measurements of TRP, KYN, KYNA, 3-HAA, 5-HAA and 5-HIAA in plasma. Baseline values were similar to those reported by others (Fukushima et al. 2009; Midttun et al. 2009; Pawlak et al. 2001; Siassi et al. 1977; Zheng et al. 2012).

We found the TRP catabolism was mainly altered in the early CPR phase. More specifically, there was an activation of the KP with decreases in circulating TRP and increases in plasma levels of KYN, KYNA and 3-HAA, especially 2 h after CPR.

Since the KP is mainly activated during inflammation (Mandi and Vecsei 2012), these results come as no surprise because activation of the inflammatory cytokine cascade, chemokine upregulation and ultimately recruitment of inflammatory leukocytes and reactive astrogliosis have been reported after CA and play major roles in the final outcome (Meybohm et al. 2009). As the KP is activated by pro-inflammatory stimuli, the reported anti-inflammatory effect of KYNA, through the inhibition of TNFα, provides an interesting feed-back mechanism in immune responses (Wang et al. 2006). Therefore in our model, KP activation might have been a consequence of the acute immune and inflammatory responses.

Kynurenine pathway activation has also been reported in the pathogenesis of vasoplegia and hypotension during septic shock (Adams Wilson et al. 2012). During systemic inflammation, the expression of indoleamine-2,3-dioxygenase (IDO), responsible for the conversion of TRP to L-KYN, is induced and contributes to the dysregulation of vascular tone (Wang et al. 2010). The increased IDO activity accounted for the greater production of L-KYN, the precursor of both KYNA and 3-HAA that directly mediates arterial relaxation (Changsirivathanathamrong et al. 2011). When this enzyme was inhibited, there was clear protection against hypotension, with reduced mortality (Jung et al. 2009). Hypotension also occurs during CPR (Krismer et al. 2006) and we cannot exclude that the raised levels of KYN, KYNA and 3-HAA at 2 h account for vascular tone alteration with systemic hypotension after successful resuscitation. Indeed in our rat model there was a significant decrease of hemodynamic parameters (e.g. mean arterial and coronary perfusion pressure) in the early CPR phase.

Interestingly, in the very early phase of CPR there was an increase in the plasma concentration of 5-HIAA, the MAO-derived end-product of serotonin (5-HT), which is in turn an oxidative metabolite of TRP along the methoxyindole pathway (see Fig. 3). This might indicate that serotonin and its metabolite contribute to the deterioration of peripheral blood flow in the early CPR phase, in accordance with recent findings of a high plasma level of 5-HIAA in subjects with metabolic syndrome in the setting of vascular injury (Fukui et al. 2012). The specific role of these TRP metabolites in the regulation of blood pressure warrant further investigation.

The KP activation attracted our attention also because mounting evidence indicates its significant role in many neurological disorders (Braidy et al. 2009; Gulaj et al. 2010; Zadori et al. 2009). The activation results in production of neuroactive and neurotoxic intermediates, closely related to the pathogenesis of depression, schizophrenia, Huntington disease, seizure, and other neuro-inflammatory and vascular diseases (Changsirivathanathamrong et al. 2011; Chen et al. 2010; Gulaj et al. 2010; Myint 2012; Stoy et al. 2005; Wang et al. 2010; Wonodi et al. 2011). Specifically, 3-HAA, besides its role in immunoregulation (Lopez et al. 2008; Morita et al. 2001), is a neurotoxin that can reduce choline acetyltransferase activity similarly to quinolinic acid, a 3-HAA derivative end-product, known to be a potent excitotoxic *N*-methyl-d-aspartate (NMDA) receptor agonist (Chen and Guillemin 2009). In addition, 3-HAA can cause oxidative stress, by increasing the production of ROS (Goldstein et al. 2000). KYNA, instead, has been identified as an endogenous NMDA and α7nicotinic acetylcholine receptor antagonist (Hilmas et al. 2001; Stone 1993) and has been shown to be neuroprotective in clinically relevant animal models of brain ischemia (Andine et al. 1988; Nozaki and Beal 1992).

In the context of CPR, increased KYNA formation might serve to counteract the negative effect of 3-HAA metabolites through the NMDA receptor in order to maintain homeostasis in the KP pathway. In our model the KYNA concentration had dropped to baseline by 4 h after CPR, while 3-HAA remained elevated, suggesting that a neurotoxic profile might persist at 4 h after CPR. Similar changes in KP metabolites have been found in acute ischemic stroke patients where elevated levels of plasmatic KYNA and 3-HAA were associated more with the initial stroke severity than long-term outcome and correlated with the stroke-induced inflammatory response (Brouns et al. 2010). Similarly, peripheral TRP catabolism started to increase before or immediately after stroke and was related to inflammatory response and oxidative stress, with a major change in 3-HAA level (Darlington et al. 2007). The fact that changes in circulating KP metabolites can be easily detected after cerebral injury indicates that kynurenines produced in the brain in response to stroke can cross the blood–brain barrier into the peripheral bloodstream (Fukui et al. 1991; Reinhard 2004).

A first limitation in the present study is the lack of investigation on rat brain recovery, that is a long-term

outcome. However this work was aimed at identifying very early modifications in the CPR setting and indeed this investigation had the capability to find correlation between the activation of the KP and different indicators of early post-resuscitation myocardial dysfunctions. A second caveat may be the use of intact animals as control rather than sham. We preferred to use intact animals to assess the physiological basal levels of TRP and metabolites. Nevertheless, animals used as healthy control, underwent anaesthesia (as the CA animals) for echocardiography and were surgically instrumented for blood collection. Indeed, any possible effects of anaesthesia on KP has been therefore taken into account.

5 Concluding remarks

Mass spectrometry-based metabolomics strategies (from untargeted to targeted) once again offer promise for the discovery of potential biomarkers in resuscitation after cardiac arrest, despite some limitations intrinsic to the technology (*e.g.* partial metabolome coverage).

Our study provides the first demonstration of KP activation early following resuscitation from cardiac arrest. Changes in TRP metabolism might account for the severity of hemodynamic instability and vascular tone alterations after successful resuscitation. Moreover our results suggests and may anticipate that imbalances between beneficial and neurotoxic kynurenine metabolites might have a role in the neurological outcome. Further studies are in progress clarify the prognostic value of TRP metabolites in the CPR outcome (Ristagno G. et al. 2012 in preparation), and if these specific metabolites offer promising features, paving the way to alternative tools for understanding the mechanisms of CA outcome.

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